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<p>(21) International Application Number: PCT/EP90/01042 (22) International Filing Date: 27 June 1990 (27.06.90) (30) Priority data: 89201735.1 29 June 1989 (29.06.89) EP (34) Countries for which the regional or international application was filed: NL et al. (71) Applicants (for all designated States except US): GIST-BRO- CADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL). PLANT GENETIC SYS- TEMS N.V. [BE/BE]; Kolonel Bourgstraat 106, Bus 6, B-1040 Brussels (BE).</p>	<p>(72) Inventors; and (75) Inventors/Applicants (for US only): QUAX, Wilhelmus, Jo- hannes [NL/NL]; Jan van Galenlaan 8, NL-2253 VB Voorschoten (NL). LAROCHE, Yves [BE/BE]; Rue Bemel 115, B-1150 Brussels (BE). VOLLEBREGT, Adrianus, Wilhelmus, Hermanus [NL/NL]; Bereklaaw 13, NL-2671 WZ Naaldwijk (NL). STANSSENS, Pa- trick [BE/BE]; De Gieylaan 13, B-9820 St. Denijs Wes- trem (BE). LAUWEREYS, Marc [BE/BE]; Wilgenstraat 22, B-2950 Haaltert (BE). (74) Agents: HUYGENS, Arthur, Victor et al.; Gist-Brocades N.V., Patents and Trademarks Department, Wateringse- weg 1, P.O. Box 1, NL-2600 MA Delft (NL). (81) Designated States: AU, BG, BR, CA, FI, JP, KR, SU, US. Published Without international search report and to be republished upon receipt of that report.</p>	
<p>(54) Title: MUTANT MICROBIAL α-AMYLASES WITH INCREASED THERMAL, ACID AND/OR ALKALINE STA- BILITY</p> <div data-bbox="516 1129 1185 1675"> </div> <p>(57) Abstract</p> <p>Thermostable and acid stable α-amylases are provided as expression products of genetically engineered α-amylase genes isolated from microorganisms, preferably belonging to the class of Bacilli. Both chemical and enzymatic mutagenesis methods are e.g. the bisulphite method and enzymatic misincorporation on gapped heteroduplex DNA. The mutant α-amylases have superior properties, e.g. improved thermostability over a broad pH range, for industrial application in starch processing and textile de-sizing.</p>		

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MUTANT MICROBIAL α -AMYLASES WITH INCREASED
THERMAL, ACID AND/OR ALKALINE STABILITY

5

INTRODUCTION

Technical Field

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The present invention relates to the field of genetic engineering and provides new DNA molecules comprising DNA sequences coding for enzymes with α -amylase activity. Specifically, mutant microbial α -amylases are disclosed having improved characteristics for use in the degradation of starch, in the desizing of textile and in other industrial processes. The disclosed α -amylases show increased thermal, acid and alkaline stability which makes them ideally suited for performing their activity under process conditions which could hitherto not be used.

Background of the invention

25 Starch consists of a mixture of amylose (15-30% w/w) and amylopectin (70-85% w/w). Amylose consists of linear chains of α -1,4-linked glucose units having a molecular weight (MW) from about 60,000 to about 800,000. Amylopectin is a branched polymer containing α -1,6 branch points every 24-30 glucose units, its MW may be as high as 100 million.

30 Sugars from starch, in the form of concentrated dextrose syrups, are currently produced by an enzyme catalyzed process involving: (1) liquefaction (or thinning) of solid starch with an α -amylase into dextrins having an average degree of polymerization of about 7-10, and (2) saccharification of the resulting liquefied starch (i.e. starch hydrolysate) with amyloglucosidase (also called glucoamylase or AG). The

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resulting syrup has a high glucose content. Much of the glucose syrup which is commercially produced is subsequently enzymatically isomerized to a dextrose/fructose mixture known as isosyrup.

- 5 α -Amylase (EC 3.2.1.1) hydrolyzes starch, glycogen and related polysaccharides by cleaving internal α -1,4-glucosidic bonds at random. This enzyme has a number of important commercial applications in, for example the sugar, brewing, alcohol and textile industry. α -Amylases are isolated from a
10 wide variety of bacterial, fungal, plant and animal sources. The industrially most important α -amylases are those isolated from Bacilli.

- In the first step of the starch degradation process, starch slurry is gelatinized by heating at relatively high
15 temperature (up to 110°C). The gelatinized starch is liquefied and dextrinized by a thermostable α -amylase in a continuous two stage process. The major process variables are starch concentration, α -amylase dose, temperature and pH. During the liquefaction-dextrinization reaction the process
20 variables must be maintained within narrow limits to achieve good conversion ratios, since serious filtration problems may arise otherwise. See, for example, L.E. Coker and K. Venkatasubramanian, in: Biotechnology, p. 165-171, Ed. P.N. Cheremisinoff, P.B. Quellette, Technicom Publ. Corp.
25 Lancaster Renn. 1985. One of the problems which frequently arises is the proper regulation of the temperature in the initial stage of the degradation process: overheating often causes denaturation of the α -amylase so that the final thinning is not sufficient. One way to avoid this is the use
30 of more thermostable α -amylases.

To that end it has been proposed to add calcium ions or an amphiphile (see e.g. EP-A-0189838), but this solution appeared to be unsatisfactory.

- There is, therefore, still substantial interest to
35 provide α -amylases with increased thermostability.

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Relevant Literature

EP-A-057976 describes the isolation of a thermostable α -amylase coding gene from B. stearothermophilus the gene is
5 cloned into a plasmid containing either a Bacillus or an E. coli origin of replication. The so obtained chimeric plasmid is used for producing α -amylase. The α -amylase gene was isolated and used without any further modification.

EP-A-0134048 describes a method for increased commercial
10 production inter alia of α -amylase, by cloning and expression of one or more α -amylase genes in industrial Bacillus strains.

EP-A-252666 describes a chimeric α -amylase with the general formula Q-R-L in which Q is a N-terminal polypeptide
15 of 55 to 60 amino acid residues which is at least 75 percent homologous to the 37 N-terminal residues of the B. amyloliquefaciens α -amylase, R is a given polypeptide and L is a C-terminal polypeptide of 390 to 400 amino acid residues which is at least 75 percent homologous to the 395
20 C-terminal residues of B. licheniformis α -amylase.

Gray et al. (J. Bacteriol., 1986, 166, 635) describe chimeric α -amylases formed of the NH₂-terminal portion of B. stearothermophilus α -amylase and the COOH-terminal portion of B. licheniformis α -amylase. Most of the hybrid enzyme
25 molecules were shown to be less stable than the parent wild-type enzymes. Furthermore none of the hybrid molecules was shown to possess improved stability properties.

None of the references cited above describes the use of single amino acid replacements to obtain novel α -amylases.

30 EP-A-0285123 discloses a method for complete mutagenesis of nucleic acid sequences. As an example mutagenesis of the B. stearothermophilus α -amylase is described. Although there is a suggestion that this method can be used to obtain B. stearothermophilus α -amylase mutants with improved stability
35 no examples are given.

SUMMARY OF THE INVENTION

The present invention provides mutant α -amylases and ways of obtaining such mutants. Said mutant α -amylases are
5 characterized in that they differ in at least one amino acid from the wild-type enzyme. Furthermore, DNAs encoding these mutants, vectors containing these DNAs in expressionable form and host cells containing these vectors are provided.

In one aspect of the invention random mutagenesis on
10 cloned α -amylase genes is disclosed. The mutated genes are expressed in a suitable host organism using a suitable vector system.

In another aspect of the invention screening methods for mutant α -amylases are described and applied. Said methods
15 yield more thermostable and more acid stable α -amylases. Furthermore, this method is used with a slight modification to obtain more alkaline stable α -amylases. The expression products of the clones so detected are isolated and purified.

In yet another aspect of the invention α -amylases are
20 provided with increased thermostability, these mutant α -amylases reduce filtration problems under application conditions of starch degradation.

In a further aspect of the invention α -amylases are provided with increased acid stability, these reduce the
25 formation of unfavourable by-products, such as maltulose, at the same time they decrease the amount of acid to be added before the reaction with amyloglucosidase. The new α -amylases possess preferably both improved properties with respect to thermostability and acid stability or with respect to both
30 thermostability and alkaline stability.

In another aspect of the invention the mutant proteins are shown to have a better performance under application conditions of starch liquefaction. The alkaline stability is especially useful for application in textile desizing.

35 These aspects will be further described in the detailed description and in the examples hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGSFigure 1: Nucleotide sequence of pMa5-8

Stanssens et al., 1987, EMBO Laboratory Course
5 Martinsried, July 1987. For description of the different
elements see text.

Figure 2: Nucleotide sequence of plasmid pPROM SPO2 insert

Construction of this vector has been described in EP-A-
10 0224294. The α -amylase amino acid sequence is depicted below
the triplets. Numbering starts from the first amino acid of
the mature protein (Kuhn et al., 1982, J. Bacteriol, 149,
372). The SPO2 promoter insert runs from position 61 to 344.

Figure 3: Nucleotide sequence of pMaTLia6

This vector was constructed from pMa5-8, the insert of
pPROM SPO2 and a synthetic DNA fragment encoding the TAC
promoter. The TAC promoter DNA fragment runs from position
3757 to position 3859. The α -amylase amino acid sequence is
20 depicted below the triplets.

Figure 4 :Restriction map of pMaTLia6

The following unique restriction enzyme sites are
available for gap construction in the α -amylase gene: BamHI,
25 SpeI, SacII, KpnI, ClaI, NarI, SalI, Th111I, XmaIII and
BstEII. Sequencing primers for all possible gaps have been
synthesized in order to enable easy determination of
mutations. Plasmid pMcTLia6 is identical with pMaTLia6 except
for the presence of an amber codon in the ampicillin gene
30 (removes ScaI site) and the absence of an amber codon in the
chloramphenicol gene (associated with the presence of a PvuII
site).

Figure 5: Outline of Bacillus/E. coli shuttle vector pBma/c

35 The (left) pMa/c section enables convenient mutagenesis
in E. coli. The (right) Bacillus subtilis cassette contains
the α -amylase gene (or any other Bacillus gene) plus a

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minimal replicon for propagation in B. subtilis. After succesful mutagenesis in E. coli the B. subtilis cassette can be circularized allowing the SPO2 promoter to move in front of the α -amylase gene upon transformation into Bacillus.

5

Figure 6: Restriction map of pBMa/c1

This vector is a specific example of the mutagenesis expression vector outlined in Figure 5.

(1) and (2): multiple cloning sites. The target gene is
10 inserted in (2). By varying the sites at (1) and (2) convenient restriction sites for gapped duplex creation can be constructed;

	FDT	: transcription terminator
	F1.ORI	: origin of replication originating from
15		phage F1
	E. coli ORI:	origin of replication from pBR322
	BLA	: ampicillin resistance gene
	CAT	: chloramphenicol resistance gene
	BAC ORI	: origin of replication of pUB110
20	KANAMYCIN	: kanamycin (neomycin) resistance gene of
		pUB110
	SPO2	: promoter of phage SPO2

Figure 7: Restriction map of pBMa/c6Lia6

25 The Bacillus licheniformis α -amylase gene was engineered into pBMa/c1 at multiple cloning site (2) of Figure 6. In this figure the SPO2 promoter is indicated by (2) and the E. coli ORI is represented by (4).

30 Figure 8: Sequence of phoA signal sequence fragment in pMa/c TPLia6

Depicted is the sequence from the EcoRI site upstream from the TAC-promoter up to the first amino acids of mature α -amylase. The phoA amino acid sequence is shown below the
35 DNA sequence.

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Figure 9: Michaelis-Menten plot for WT and 2D5 α -amylase

This plot shows the initial rate of enzyme activities vs. substrate concentration for WT and 2D5 α -amylase. Assay conditions are described in Example 8.

5

Figure 10: Thermoinactivation of WT and D7 α -amylase

This plot shows the half life time of both WT and D7 α -amylase as a function of the Ca^{2+} concentration at pH 5.5 and 90.5°C.

10

Figure 11: Thermoinactivation of WT and D7 α -amylase

As in Figure 10 except for the pH which is 7.0.

Figure 12: Thermoinactivation of WT and 2D5 α -amylase

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This plot shows half life times of both WT and 2D5 α -amylase as a function of Ca^{2+} concentration at pH 7.0 and 95°C.

Figure 13: Thermoinactivation of WT and D7 α -amylase as a function of pH

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Figure 14: Thermoinactivation of WT and 2D5 α -amylase as a function of pH

Figure 15: DE vs final pH measured after liquefaction at 110°C

DETAILED DESCRIPTION OF THE INVENTION

30 By the term "exhibits improved properties" as used in connection with "mutant α -amylase" in the present description we mean α -amylases which have a higher enzymatic activity or a longer half-life time under the application conditions of starch liquefaction, textile desizing and other industrial
35 processes.

With "improved thermostability" we mean that the mutant enzyme retains its activity at a higher process

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temperature, or that it performs longer at the same temperature than the wild-type enzyme from which it originates.

With "improved acid (or alkaline) stability" we mean
5 that the mutant enzyme performs better at lower (or higher) pH values than the wild-type enzyme from which it was derived.

It is to be understood that the improved properties are caused by the replacement of one or more amino acids.

10

Chromosomal DNA may be isolated from an α -amylase containing microorganism. Preferably a microorganism is used belonging to the genus Bacillus, more preferably B. licheniformis, still more preferably B. licheniformis T5
15 is used (see EP-A-134048). The chromosomal DNA is digested with a suitable restriction enzyme and cloned into a vector. A number of possible ways of selection can be used e.g. hybridization, immunological detection and detection of enzymatic activity. The choice of the vector used for cloning
20 the digested chromosomal DNA will depend on the selection method available. If hybridization is used no special precautions are needed. However, if detection is immunological or based on enzymatic activity the vector will have to contain the proper expression signals. The actual
25 detection of clones containing α -amylase was performed on starch containing agar plates. After growth and incubation with I_2 vapor halos are detected around positive clones. As a next step the sequence of the gene is determined. The derived amino acid sequence is used for comparison with other known
30 α -amylase sequences to give a first impression of important amino acids (e.g. active-site, Ca^{2+} binding, possible S-S bridges). A better indication is obtained when the 3D-structure is determined. Since this is very laborious oftentimes another approach is used. In the absence of a 3D-
35 structure prediction programs for determining the secondary structural elements (e.g. α -helix, β -sheet) are successfully used eventually the tertiary structural elements e.g. β -

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barrel are determined. For a review see Janin, J. and Wodack, S.J., Prog. Biophys. molec. Biol. 1983, 42, 21-78.

Valuable amino acid replacements can be envisioned. The stability of a protein structure is determined by the net
5 difference in free energy between the folded and unfolded conformations of the protein. Since the proline residue is restricted to fewer conformations than the other amino acids the configurational entropy of unfolding a protein is decreased (and stability thereby increased) when an amino
10 acid is replaced with proline. Another useful substitution is the glycine to alanine replacement. Residues such as threonine, valine and isoleucine with branched β -carbons restrict the backbone conformation more than non-branched residues.

15 Since a part of the thermostability of certain proteins is due to salt bridges it may be advantageous to introduce lysine and arginine residues (Tomozic S.J. and Klibanov A.M., J. Biol. Chem., 1988, 263 3092-3096). Moreover replacement of lysine by arginine residues may improve the
20 stability of salt bridges since arginine is able to form an additional H-bond. For a review see Wigby, D.B. *et al.* Biochem. Biophys. Res. Comm. 1987, 149, 927-929. Deamidation of asparagine and glutamine is mentioned to cause a serious disruption of the enzyme structure, replacement with non-
25 amide residues may avoid this disruption. Amino acid replacements are best made by mutagenesis at the DNA level.

In principle mutagenesis experiments can be performed immediately on isolated clones. However, the insert is preferably cloned in a mutagenesis/expression vector. Random
30 mutagenesis is possible and so is site-directed mutagenesis. In view of the huge amount of mutated clones of the former method, and since no 3D-structure of α -amylase is known to make possible an educated guess for site-directed mutagenesis we decided to perform "random" mutagenesis in specific
35 regions.

The following is a possible approach for practising the present invention.

- 10 -

First the gene is modified by the introduction of "silent" restriction sites. Introduction of non-silent restriction sites is also possible. This makes possible the deletion of specific regions of the gene. Secondly the gene
5 is cloned in a phasmid. This combination of a phage and a plasmid makes easy the production of single stranded DNA. Other ways of obtaining single stranded DNA are also possible. By hybridizing melted double-stranded vector (plus insert) DNA with a vector/insert combination containing a gap
10 in the insert, gapped heteroduplex DNA was obtained (for a detailed description see Morinaga, Y et al. 1984, Biotechnology, 2, 636).

The gap is used for chemical or enzymatic mutagenesis. Preferably we used the bisulphite method (Folk and
15 Hofstetter, Cell, 1983, 33, 585) and an enzymatical misincorporation method are used (modified version of Lehtovaara et al., Prot. Eng., 1988, 2, 63). These methods can be applied in such a way that every single nucleotide in the gap is replaced by all three other nucleotides
20 (saturation mutagenesis). The latter method can be applied in several ways. In one of them a synthetic primer is hybridized to the gap. Subsequently an extension reaction is performed in which the deoxynucleotide complementary to the first deoxynucleotide 3' from the primer is missing. In principle
25 all three of the other deoxynucleotides can thus be incorporated. This can be achieved either by using a mix of three deoxynucleotides or by using three separate reactions each containing only one deoxynucleotide. Another way of applying the method yields random clones. Here, four separate
30 reactions are set up each of them containing one limiting deoxynucleotide. This gives second strands that stop before every single nucleotide. The subsequent steps can be performed as described above. Both the bisulphite and the enzymatic mutagenesis method were employed to obtain mutants.
35 For testing the enzymatic properties it may be convenient to express the cloned genes in the same host as that used during mutagenesis experiments. In principle this

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can be any host cell provided that suitable mutagenesis/expression vector systems for these cells are available. For the most part E. coli is very convenient to work with, for example E. coli WK6. After growth of the colonies in microtiterplates samples from the wells of these plates are spotted on agar plates supplemented with starch and buffered at different pH values. Positive clones can be detected by halo formation. Screening with appropriate buffers can be used to select for thermostability, acid stability, alkaline stability, saline stability or any other stability that can be screened for.

Suitable host strains for production of mutant α -amylases include transformable microorganisms in which the expression of α -amylase can be achieved. Specifically host strains of the same species or genus from which the α -amylase is derived, are suited, such as a Bacillus strain. Preferably an α -amylase negative Bacillus strain is used more preferably an α -amylase and protease negative Bacillus strain.

For example B. licheniformis T9 has been used to produce high amounts of mutant α -amylases.

Preferably, the α -amylases being produced are secreted into the culture medium (during fermentation), which facilitates their recovery. Any suitable signal sequence can be used to achieve secretion.

The expressed α -amylase is secreted from the cells and can be subsequently purified by any suitable method. Gelfiltration and Mono Q chromatography are examples of such methods. The isolated α -amylase was tested for thermo-inactivation at different Ca^{2+} concentrations (0.5 - 15 mM) and over a wide pH range (5.5 - 8.0). Tests were also performed under application conditions. Specifically mutant α -amylase was tested under conditions of starch liquefaction at pH 5.5 and 5.25. Furthermore, applications for textile desizing have been tested.

The properties of some of the mutants that are screened will be better suited under the desired performance conditions.

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The present invention discloses α -amylases with increased thermostability, improved acid stability and improved alkaline stability. Generally the number of amino acid replacements is not important as long as the activity of the mutated protein is the same or better than that of the wild-type enzyme. Mutant α -amylases differ in at least one amino acid from the wild-type enzyme, preferably the mutants differ in from 1 to 10 amino acids. Specific mutants with improved properties include mutant α -amylases containing one or more amino acid replacements at the following positions 111, 133 and 149 (numbering is in accordance with the B. licheniformis α -amylase). Among the preferable amino and replacements are Ala-111-Thr, His-133-Tyr and Thr-149-Ile.

Such mutant enzymes show an improved performance at pH values below 6.5 and/or above 7.5. The performance is also increased at high temperatures leading to an increased half-life-time at for example temperatures of up to 110°C.

Many of the available α -amylase products are obtained from bacterial sources, in particular Bacilli, e.g. B. subtilis, B. licheniformis, B. stearothermophilus, B. coagulans and B. amyloliquefaciens. These enzymes show a high degree of homology and similarity (Yuuki et al., J. Biochem., 1985, 98, 1147; Nakajima et al., Appl. Microbiol. Biotechnol., 1986, 23, 355). Therefore knowledge of favourable mutations obtained from one of these α -amylases can be used to improve other amylases. The present invention provides an approach for obtaining such knowledge.

Following is a description of the experimental methods used and examples to illustrate the invention. The examples are only for illustrative purpose and are therefore in no way intended to limit the scope of the invention.

EXPERIMENTAL

35

Materials and Methods

1. General cloning techniques

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Cloning techniques were used as described in the handbooks of T. Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory; F.M. Ausubel et al., 1987, 5 Current Protocols in Molecular Biology, John Wiley & Sons Inc., New York; B. Perbal, 1988, A practical Guide to Molecular Cloning, 2nd edition, John Wiley & Sons Inc., New York. These handbooks describe in detail the protocols for construction and propagation of recombinant DNA molecules, 10 the procedures for making gene libraries, the procedures for sequencing and mutating DNA and the protocols for the enzymatic handling of DNA molecules.

15 2. Chemical mutagenesis

Cloned DNA may be treated in vitro with chemicals in order to introduce mutations in the DNA. If these mutations are directed to amino acid encoding triplet codons a mutated 20 protein can be produced by the mutated cloned DNA. A method for chemical mutagenesis with the aid of sodium bisulfite is described by Shortle and Botstein (Methods Enzymol., 1983, 100, 457). A preferable method is described by Folk and Hofstetter (Cell, 1983, 33, 585). Other methods for 25 mutagenesis are described by Smith, Ann. Rev. Genet., 1985, 19, 423. A particularly useful protocol is described by Ausubel et al., ibid.

30 3. Mutagenesis on gapped-duplex DNA

A method based on the gapped-duplex approach (Kramer et al., 1984, Nucl. Acids Res. 12, 9441) and a phasmid (plasmid/phage hybrid) was used. Essentially the method rests 35 on a gapped duplex DNA intermediate consisting of a gapped strand (-strand) containing a wild-type antibiotic resistance marker and a template strand (+ strand) carrying an amber

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mutation in the gene conferring resistance to the antibiotic. After annealing, the mutagenic oligonucleotide becomes incorporated in the gapped strand during in vitro gap-filling and sealing reaction. The resultant molecules are used to
5 transform a mismatch repair deficient (Mut S) host in which the linkage between the intended mutation and the antibiotic resistance marker is preserved. The mixed phasmid population, isolated from this strain, is then allowed to segregate in a suppressor negative host strain. Transformants are plated on
10 antibiotic containing medium, thus imposing a selection for progeny derived from the gapped strand.

The twin vector system pMa/c5-8, which was described by P. Stanssens *et al.* (Nucl. Acids Res., 1989, 17, 4441) is composed of the following elements:

- 15 pos 11-105 : bacteriophage fd, terminator
- pos 121-215 : bacteriophage fd, terminator
- pos 221-307 : plasmid pBR322 (pos 2069-2153)
- pos 313-768 : bacteriophage f1, origin of replication
(pos 5482-5943)
- 20 pos 772-2571 : plasmid pBR322, origin of replication
and β -lactamase gene
- pos 2572-2685: transposon Tn903
- pos 2519-2772: tryptophan terminator (double)
- pos 2773-3729: transposon Tn9, chloramphenicol acetyl
25 transferase gene
- pos 3730-3803: multiple cloning site

The sequence is depicted in Figure 1.

- 30 In the pMa type vector nucleotide 3409 is changed from G to A, while in the pMc type vector nucleotide 2238 is changed from G to C, creating amber stopcodons in the acetyl transferase gene and β -lactamase gene, respectively, rendering said genes inactive.

35

All sequences referred to were obtained from Genbank (TM) (release 54), National Nucleic Acid Sequence Data Bank,

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NIH USA. Plasmid pMc5-8 has been deposited under DSM 4566. To perform mutagenesis the target DNA fragment is cloned into the multiple cloning site of pMa5-8. Subsequently a gapped duplex between pMa5-8 containing the target DNA and pMc5-8 is constructed.

The single strand gap, consisting of the target DNA, can be subjected to mutagenesis with a mutagenic oligonucleotide, with long synthetic oligonucleotides, with a low level of misincorporated nucleotides, with chemicals or with enzymatic misincorporation of nucleotides also random mutagenesis PCR can be applied. For a detailed description see Ausubel *et al.*, *ibid.* or Perbal, *ibid.* As an alternative to in vitro mutagenesis one can use in vivo mutagenesis either with the aid of UV-light or chemicals or by the application of an *E. coli* mutator strain (Fowler *et al.*, *J. Bacteriol.*, 1986, 167, 130).

Mutagenic nucleotides can be synthesised using apparatus obtainable from Applied Bio Systems.

20

4. Random mutagenesis by enzymatic misincorporation of nucleotides

A pMa/pMc gapped duplex can be subjected to primer extension and misincorporation mutagenesis as originally described by Shortle *et al.* (Proc. Natl. Acad. Sci. USA, 1982, 79, 1588) by B.C. Cunningham and J.A. Wells (Prot. Eng., 1987, 1, 319) a modification of this procedure is described by Lehtovaara *et al.*, (Prot. Eng., 1988, 2, 63).

This method is based on controlled use of polymerases. Four populations of DNA molecules are first generated by primer elongation of a gapped duplex of pMa/pMc so that they terminate randomly, in the gap, but always just before a known type of base (before A, C, G or T, respectively). Each of four populations is then mutagenized in a separate misincorporation reaction where the correct base can now be omitted. In this way all types of base substitution mutations

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can be generated at every position of the gap. The use of sequenase (TM) (U.S. Biochemical Corporation) was preferred to the use of Klenow polymerase. Moreover MoMuLV reverse transcriptase was used instead of A.M.V. reverse

5 transcriptase, which was used by Lehtovaara et al. (ibid).

To ensure single site substitutions we have introduced the following modification to the protocol described by Lehtovaara et al., ibid. In the reverse transcriptase buffer not three but only one misincorporating nucleotide is
10 present. For instance the A-specific limited base elongation mixture is incubated in three separate reactions with 250 μ M dCTP, 250 μ M dGTP and 250 μ M dTTP, respectively. For a complete set of 4 base specific limited elongation mixtures a total set of 12 separate misincorporation reactions is
15 carried out. After 1.5 hour incubation at 42°C a chase of all four deoxynucleotides in a concentration of 0.5 mM is added and the reactions are further incubated for at least 20 minutes at 37°C. Samples are then further processed according to Lehtovaara et al. (ibid.), with the modification that no
20 counterselection to an uracil-containing DNA strand but a counterselection based on the pMa/c vector was applied.

5. Production of mutant α -amylases

25

Transformants of E. coli strain WK6 (Zell, R. and Fritz, H.J., EMBO J., 1987, 6, 1809), containing an expression vector, harboring any one of the α -amylase constructs, were inoculated in TB medium (10 ml) at 30°C. TB
30 medium consisted of 0.017M KH_2PO_4 , 0.072M K_2HPO_4 , 12 g/l Bactotryptone, 24 g/l Bacto yeast extract, 0.4% glycerol and an antibiotic (ampicillin with pMa or chloramphenicol with pMc constructs). Samples of the culture were used to inoculate 250 ml TB in 2 liter flasks. At an OD_{600} of
35 10 - 12, 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) was added and incubation continued for another 12 - 16 hours.

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6. Purification of mutant α -amylases

- 5 The cells were harvested by centrifugation and
resuspended in buffer containing 20% sucrose at 0°C. After a
second centrifugation the cells were resuspended in cold
water. Cell debris was removed by a third centrifugation and
the supernatant was brought to pH 8.0 with 20mM TRIS buffer.
- 10 CaCl₂ was added to a final concentration of 50mM. The
material was heat-treated for 15 min. at 70°C and the
insoluble material removed by centrifugation. The supernatant
was filtered through 0.22 μ Millipore filter and concentrated
to 1/10th of the starting volume.
- 15 Further purification was achieved using gelfiltration
(on TSK HW-55- Merck) and Mono Q chromatography. Before
chromatography on Mono S the pH, of the enzymatic activity
containing fractions, was adjusted to 4.8 using sodium
acetate. α -amylase was eluted with 250mM NaCl. To avoid
- 20 inactivation the pH was immediately adjusted to 8.0.

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Examples

Example 1

Molecular cloning of *Bacillus licheniformis* α -amylase gene

5 Chromosomal DNA isolated from *Bacillus licheniformis* T5 (EP-A-134048; CBS 470.83) was digested with restriction enzyme *EcoRI* and ligated into the *EcoRI* site of pUB110 (Gryczan, T.J., et al., J. Bacteriol, 1978, 134, p 318). The
10 ligation mixture was transformed into *Bacillus subtilis* 1A40 (Bacillus Genetic Stock Center). Neomycine resistant colonies were tested for α -amylase production on HI agar plates (DIFCO) supplemented with 0.4 g/l starch (Zulkowsky starch, Merck). After growth and incubation with I₂ vapor, a positive
15 colony producing a large clearing halo was selected for further characterization. The plasmid isolated from this positive colony was shown to contain a 3.4 kb *EcoRI*-*EcoRI* fragment originating from *Bacillus licheniformis* T5. This plasmid was named pGB33 (EP-A-134048; CBS 466.83). The α -
20 amylase encoding insert was ligated to a synthetic Shine-Dalgarno sequence and the bacteriophage SPO2 promoter resulting in plasmid pProm SPO₂ (see EP-A-0224294; CBS 696.85). The nucleotide sequence of the insert of pProm SPO₂ as determined by the method of Sanger (Proc. Natl. Acad. Sci.
25 U.S.A., 1977, 74, 6463) is shown in Figure 2. The sequence shows a single large open reading frame encoding an α -amylase, which is virtually identical to the α -amylase sequence of *Bacillus licheniformis* as determined by Yuuki et al. (ibid). The first 29 amino acids are a signal sequence
30 which is cleaved off during secretion of the α -amylase. Numbering of amino acids throughout this application refers to the numbering according to the mature protein.

The Yuuki sequence differs at the following positions:
at position 134 an Arg is present instead of Leu; at position
35 310 a Ser is present instead of Gly; at position 320 an Ala is present instead of Ser.

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Example 2Construction of mutagenesis/expression vectors pMatLia6

- 5 Plasmid pPROM SPO₂ was digested with EcoRI and BclI and the 1.8 kb EcoRI-BclI insert was purified and cloned into EcoRI-BamHI digested pMa5-8. This pMa5-8 vector was before-hand provided with a modified multiple cloning site. The BamHI-HindIII fragment running from position 3767 to position
- 10 3786 in Figure 1 was exchanged for a synthetic DNA sequence as it reads from position 5647 to 5660 in Figure 3. This was carried out to render some restriction sites within the α -amylase gene unique. The resulting α -amylase containing pMa5-8 derivative was digested with EcoRI and BamHI and ligated to
- 15 a synthetic DNA fragment carrying a copy of the TAC promoter (De Boer et al., Proc. Natl. Acad. Sci. U.S.A., 1983, 80, 21). The sequence of this synthetic DNA fragment is depicted together with the final α -amylase mutagenesis/expression vector pMatLia6 in Figure 3 from position 3757 to position
- 20 3859. This final α -amylase mutagenesis/expression vector was completed by the introduction of several silent restriction sites which are intended to produce gaps in the α -amylase gene during mutagenesis experiments (Figure 4). For this purpose the following mutations have been made using site-
- 25 directed oligonucleotide mutagenesis:
- a SpeI site has been introduced by a silent mutation:
T49T and S50S
ACG --> ACT AGC --> AGT
- 30
- a NarI site has been introduced by the silent mutation:
A269A
GCG --> GCC
- 35
- A BstE II site has been introduced just downstream from the TAG stop codon

- 20 -

TAGAAGAGC --> TAGGTGACC

This α -amylase mutagenesis vector pMatLia6 is suited for mutagenesis with the gapped duplex method. Double stranded pMatLia6 DNA prepared by digestion of suitable restriction enzymes has been annealed to single stranded pMcTLia6 DNA.

The resulting single stranded gaps have been subjected to site-directed mutagenesis, to chemical mutagenesis and to random enzymatic mutagenesis as described in the experimental section.

The availability of the TAC promoter in front of the α -amylase gene enables the inducible expression of α -amylase in E. coli by addition of IPTG.

Plasmid pMatLia6 in E. coli WK6 was deposited as CBS 255.89 on June 2nd, 1989.

Example 3

Construction of a Bacillus/E. coli shuttle vector for mutagenesis and expression

This vector enables mutagenesis of an inserted gene in E. coli and immediate expression in Bacillus. The strategy chosen for the construction of the vector was to combine a pUB110 derivative (Gryczan, *ibid.*) with the pMa/c twin vector system in such a way that:

1. The B. subtilis cassette can be removed by a single restriction/religation experiment.
2. Different α -amylase genes and different promoters can be easily cloned in this vector.
3. After recircularisation the cloned gene will be under control of a suitable Bacillus promoter.
4. During mutagenesis in E. coli the Bacillus promoter and the structural α -amylase gene are physically separated preventing a possible lethal accumulation of α -amylase in E. coli.

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A schematic drawing of the shuttle vector is shown in Figure 5. The structure of the final version of the vector pBma/c1 is depicted in Figure 6. Vector pBma1 has been deposited under number CBS 252.89, on June 2nd, 1989. The

5 vector has been constructed as follows:

- The EcoRI-SnaBI fragment of pUB110 carrying the REP-gene and the Neo^R gene was purified and cloned into EcoRI-SmaI digested pUC8.
- 10 - The EcoRI-HindIII fragment of this pUC8 derivative was cloned into EcoRI-HindIII digested pMa5-8 resulting in plasmid pMa5-80.
- The BamHI-XbaI polylinker fragment was substituted by a synthetic fragment of DNA encoding the SPO₂ promoter of bacteriophage SPO₂ (Williams *et al.*, J. Bacteriol., 1981, 146, 1162) plus restriction
- 15 recognition sites for SacII, ApaI, XhoI, SacI, BglI, MluI and XbaI.
- The unique EcoRI site of pMa5-80 was used to insert a polylinker fragment constituting the following
- 20 recognition sites: EcoRI, SmaI, SacI, EcoRV, SphI, KpnI, XbaI and HindIII

For specific purposes derivatives pBma/c2 and pBma/c6 have been developed out of pBma/c1.

25

- In pBma/c2 the EcoRI-HindIII polylinker of pBma/c1 has been replaced by the corresponding polylinker of pUC19.
- In pBma/c6 in addition the SacII site in the right
- 30 polylinker of pBma/c1 has been removed by a Klenow reaction.

Site directed mutagenesis on the B. licheniformis α -amylase gene was performed after construction of pBma/c6

35 Lia6. This vector was constructed by ligating the BamHI-HindIII fragment isolated from pMaTLia6 into the above mentioned pBma/c6 which was cleaved by BamHI and HindIII. The

- 22 -

resulting plasmid (Figure 7) can be used to construct gapped duplexes for mutagenesis in E. coli.

The resulting mutants have been expressed in Bacillus subtilis 1A40 (BGSC 1A40) after restriction with SacI,
5 religation and transformation according to Chang and Cohen (Mol. Gen. Genet., 1979, 168, 111).

Example 4

10 Expression in E. coli of correctly matured Bacillus licheniformis α -amylase

Characterization of the α -amylase produced by pMatLia
6 (Example 2) showed that a portion of the α -amylase was
15 incorrectly processed during secretion. NH₂-terminal
sequencing revealed an extra Alanine residue for α -amylase
produced in E. coli WK 6.

Although we have no indication that this will give
different properties to the amylase we have replaced the α -
20 amylase signal sequence by the alkaline phosphatase PhoA
signal sequence. To this end a mutagenesis experiment was
carried out so as to introduce a FspI restriction site in
pMatLia 6 at the junction of the signal peptide and the
mature α -amylase. After FspI and BamHI digestion a synthetic
25 DNA fragment encoding the phoA signal sequence (Michaelis et
al. J. Bacteriol., 1983, 154, 366) was inserted. The sequence
of this construction is shown in Figure 8. α -Amylase produced
by pMa/CTPLia6 was shown to possess the correct NH₂-terminal
sequence.

30

Example 5

Screening for stable α -amylase

A. Screening for acid-stable α -amylase mutants

35

α -Amylase mutants, that perform better or worse at low
pH than the wild-type α -amylase, can be selected by

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comparison of halo's on starch plates buffered at different pH values after staining the starch with an iodine-solution.

Method:

5

1. Growth

Possible mutants are grown in microtiterplates. The growth medium is 250 μ l Brain Heart Infusion broth (DIFCO). The following additions are made:

10 chloramphenicol 50 μ g/ml
I.P.T.G. (SIGMA) 0.2 mM
CaCl₂ 2 mM

Colonies are picked from agar plates with sterile toothpicks and inoculated in separate wells (96) of a microtiterplate.

15 In each plate 4 wild-type colonies are included as a control.

These microtiterplates are placed at 37°C for 40 hours without shaking.

2. Plate test

20 After this time period, in which the α -amylase is produced, 5 μ l samples are taken from each well and spotted on 2 different types of agar plates (144 x 140 mm). The first type is a rich Heart-Infusion agar plate (DIFCO) + 0.4% starch (Zulkowsky starch-Merck) + chloramphenicol 50 μ g/ml.
25 After incubation at 37°C for 16 hours this plate serves as a storage for mutants.

The second type of plate is the actual screening plate, it contains:

Bacto agar (DIFCO) 1.5%

Zulkowsky starch 0.2%

30 Agar and starch are dissolved in synthetic tap water (STW). This is: demineralised water +

CaCl₂ 2 mM

MgCl₂ 1 mM

NaHCO₃ 2.5 mM

35 BSA 10 μ g/ml

- 24 -

The screening plates are buffered by a 100-fold dilution of a 5 M stock potassium acetate buffer solution in this medium. pH values of the stock solutions are 4.80; 5.0 and 5.2 at room temperature. Final pH values in the agar plate when measured are somewhat lower than those of the stock solutions. From each well 5 μ l of culture is spotted on 3 screening plates with different pH values.

The pH-range is chosen in such a way that there is little or no activity left for the wild-type α -amylase on the plate with the lowest pH-value.

3. Colouring

The screening plates are incubated for 2 hours at 55°C. After this period an I₂ solution is poured over the plates. 10 x I₂ solution contains 30 g I₂ and 70 g KI per liter.

The amount of clearance of the spots is correlated with the residual α -amylase activity at that pH value. Those mutants that perform better than the wild-type controls are selected for a second round of screening. Wild-type halo's are very reproducible in this experiment.

4. Second screening

Positive mutants are picked from the rich plate and purified on fresh HI plates + chloramphenicol. 4 single colonies are picked from each mutant and they are tested again in a similar way as in the first screening. In addition serial dilutions of these cultures are made with STW and these dilutions are spotted on neutral pH screening plates (pH = 7.0). Comparison with wild-type cultures enables one to decide if the better performance at low pH is due to an overall better α -amylase production or to intrinsically more stable α -amylase.

The mutants that "survive" the second screening are characterized by determining the nucleotide sequence of that part of the gene that was subjected to mutagenesis.

- 25 -

B. Screening for alkali stable α -amylase

Screening for alkali stable α -amylases is performed in a manner similar to the one used for acid stable α -amylase.

- 5 After growth in microtiter plates 5 μ l samples are taken from each well and spotted onto a storage plate and onto the actual screening plate. The latter is composed of:

	Bacto Agar (DIFCO)	1.5%
10	Zulkowsky starch	0.2%

and completed with demineralized water plus

	CaCl ₂	2 mM
15	MgCl ₂	1 mM
	NaHCO ₃	2.5 mM
	BSA	10 μ g/ml

- The screening plates are buffered with 50 mM
20 carbonate/bicarbonate buffer, pH values are 9.0, 9.5 and 10.0. The pH range is chosen in such a way that there is little or no activity of the wild-type α -amylase at the highest pH value. After 2 hours incubation at 55°C an I₂ solution is poored over the plates. Those mutants that give a
25 better halo than the wild-type enzyme are selected for a second round of screening. This second round of screening is performed in a similar fashion as the screening for the acid stability.

30

C. Screening for thermostable α -amylase mutants

- α -Amylase mutants that perform better or worse at high temperature than the wild-type α -amylase, can also be
35 selected by comparison of halo's on starch plates caused by the residual amylase activity in the culture broths after heating.

- 26 -

Method:

1. Mutants are grown in the same way as for the pHscreening.
- 5 2. The mutants are replicated on HI agar plates as for the pH-screening.
- 10 3. The separate wells of the microtiterplates were closed with disposable caps (Flow laboratories) to prevent evaporation of the culture broths during the heating step.
- 15 4. Microtiterplates were heated in a waterbath for 1 hour at 95°C. After heating the microtiterplates were placed in a centrifuge for collecting the total sample on the bottom of the microtiterplate.
- 20 5. Screening for thermostable mutants was done as follows:
From each well 5 µl of culture was spotted on neutral screeningplates (See pH-screening). These plates were incubated for 1 hour at 55°C.
25 After staining the starch with the iodine solution mutants and controls can be screened for residual α-amylase activity by comparing clearance of the spots (halo's).
In case the residual activity of the controls is too high, serial dilutions must be made and spotted on the
30 screening plate to be able to discriminate for mutants that are more thermostable than the wild-type enzyme.
- 35 6. Possible interesting mutants are tested further as was done in the pH-screening method.

A combination of screening type A or B with type C can be applied if a combination of properties is desired. For

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instance after the first round of screening for alkali stable α -amylase, a second round of screening for thermostability can be performed. Those mutants that score positive in both tests may be selected as candidates exhibiting a combination of desired properties.

Example 6

Bisulphite mutagenesis of pMatLia6

10

Single stranded DNA of pMatLia6 was annealed with SacII-ClaI digested pMCTLia6 in order to obtain a heteroduplex with a gap running from position 4315 to 4569 (Figure 3). This heteroduplex was subjected to bisulphite mutagenesis (see experimental).

15

After transformation into E. coli WK6 mut S (Zell, R. and Fritz H.J., *ibid*) and selection on chloramphenicol containing agar plates (50 μ g/ml) plasmid pools were isolated and transformed into E. coli WK6. E. coli WK6 Mut S was deposited as CBS 472.88, E. coli WK6 was deposited as CBS 473.88. Resulting transformants were grown in BHI medium (DIFCO) containing 2.0 mM CaCl_2 , 50 μ g/ml chloramphenicol and 0.20 mM IPTG (SIGMA) during 40 hours at 37°C in microtiter wells without shaking. Screening for pH stable mutants was carried out as described in Example 5.

25

About 300 Cm^R transformants were screened. The mutation frequency as determined by DNA sequencing was on average 0.4 mutation/molecule over the gap. One acid stable mutant, D7, was identified after the pH screening. Sequencing of this mutant revealed mutation H133Y originating from a mutation of the encoding triplet from CAC to TAC.

30

Mutant D7 was also found positive in the thermostability screening assay (Example 5).

DNA sequencing was performed on single stranded DNA with a specific oligonucleotide designed to prime just before the SacII-ClaI fragment. In a separate mutagenesis experiment 1000 Cm^R transformants were screened. Another acid stable

35

- 28 -

mutant, 2D5, was identified after the pH screening. This mutant has the following mutations:

H133Y CAC --> TAC

T149I ACA --> ATA

- 5 Bisulphite mutagenesis has been applied in a similar manner as just described on the ClaI-SalI gap which runs from position 4569 to position 4976 of Figure 3. About 300 Cm^R transformants were screened (mutation frequency 0.6 mutations/molecule). No acid stable transformants were found.
- 10 A number of acid labile mutants were found. Among these acid labile mutants some may have a shifted pH spectrum resulting in a more alkaline stable phenotype.

Example 7

15

Enzymatic mutagenesis of pMatLia6

- Single stranded pMatLia6 (Figure 4) was annealed with ClaI-SalI digested pMcTLia6 in order to obtain a heteroduplex
- 20 running from position 4569 to 4976 (Figure 3). The gapped duplex was subjected to enzymatic misincorporation mutagenesis as described in the experimental section.

- A sample obtained after dATP-limited primer elongation was split in three parts and incubated in the presence of
- 25 reverse transcriptase with dCTP, dGTP and dTTP, respectively. After incubation at 37°C for 10 minutes a chase with all four dNTP's and Klenow polymerase was given T4-DNA ligase was added to finish the elongation to completely double stranded molecules.

- 30 These molecules were transformed into E. coli WK 6 Mut S and plasmid pools were recovered. These plasmid pools were subsequently transformed into E. coli WK 6 and the colonies were selected on chloramphenicol (50 µg/ml) containing agar plates. Resulting mutants were screened for stability of α-
- 35 amylase as described in Example 5.

In another experiment the SpeI-SacII gap was subjected to limited primer elongation with dATP, dCTP, dGTP and dTTP,

- 29 -

respectively. These primer pools were mutagenized by misincorporation (see experimental). 100 Cm^R transformants were tested on pH plates (Example 5) and mutant M29 was identified as more stable at low pH. The sequence of the

5 mutation was determined: A111T GCG --> TCG

Example 8

Properties of stable mutants

10

Two of the mutants obtained from the bisulphite mutagenesis experiments were further characterized. As described before DNA sequencing suggested the following amino acid replacements;

- 15 - D7 contained a tyrosine at position 133 instead of a histidine (D7 = H133Y),
 - 2D5 contained the D7 mutation and in addition threonine 149 was replaced by isoleucine (2D5 = H133Y, T149I).

20 a) Measurement of enzymatic activity

The enzymatic activity of B. licheniformis α -amylase WT and mutants was measured using 4-nitrophenyl-maltopentaoside (4NP-DP5) as a substrate, 4 nitrophenol and maltopentaose are

25 formed, this reaction can be followed by measuring the change in OD 405. The assay was performed at 35°C in 50mM MOPS, 50mM NaCl, 2mM CaCl₂ (PH 7.15) and 0-1mM 4NP-DP5. Initial rates were measured and E-nitrophenol was taken as 10,000 l/M/cm. Figure 9 shows the results for WT and 2D5 α -

30 amylases. Vmax and Km were calculated and are given in Table 1.

	Vmax(μ mol/min/mg)	Km(mM)
WT	66.7 \pm 0.9	0.112 \pm 0.005
35 2D5	66.3 \pm 0.7	0.119 \pm 0.004

Table 1

- 30 -

Table 1 clearly shows that the mutations of α -amylase 2D5 do not influence the enzymatic activity in a substantial way.

5 b) Influence of Ca^{2+} on the thermoinactivation

Heat inactivation experiments were performed for WT, D7 and 2D5 at varying calcium concentrations. The procedure was as follows :

10

1) Demetallization

Enzyme (2 - 3 mg/ml) dialyzed for 24 hrs against

3 x 1 L 20 mM MOPS

5 mM EDTA

15

5 mM EGTA pH 7.0

3 x 1 L 20mM MOPS pH 7.0

2) Remetallization

20

- 500 μl buffer 100 mM (e.g. MES, MOPS, EPPS)*

- 145 μl demetallized enzyme (e.g. 2.15 mg/ml)

- 100 μl CaCl_2 (100, 50, 30, 20, 10, 5 or 2.5 mM)

- x μl K_2SO_4 (100 mM)

- (255-x) μl H_2O

25

[CaCl_2] final (mM)	[K_2SO_4] final (mM)
0,25	14,75
0,5	14,5
1	14
2	13
3	12
5	10
10	0

30

0,25

14,75

0,5

14,5

1

14

2

13

3

12

35

5

10

10

0

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- * - pH MES e.g. 6.77 at room temperature will give 6.0 at 90°C ($pK_a \text{ } 6.15 \text{ } pK_a/^{\circ}C = -0.011$)
- pK_a were from Table of Merck
(Zwitterionische Puffersubstanzen)

5

3) Heat-inactivation

1 ml enzyme solution preincubated at room temperature was heated at 90.5°C or 95°C in closed Pierce-vials (teflon coated-seals) at a concentration of about 0.2 mg/ml. 50 μ l samples were withdrawn at regular intervals between 0 and 6 hrs with a syringe and cooled on ice. Residual activities have been determined with 4NP-DP5 (0.5mM).

Half lives were determined using a single exponential decay fitting program (GRAPHPAD).

Figures 10 and 11 show the half life times of WT and D7 α -amylases at pH 5.5 and 7.0 respectively as a function of the Ca^{2+} concentration at 90.5°C. The Ca^{2+} dependence of 2D5 has only been determined at pH 7.0 at 95°C (Figure 12). It can also be seen that the Ca dependence of the mutants is not different from that of the WT.

c. Thermostability of mutant α -amylases at different pH values

The pH dependence of thermoinactivation for both D7 and 2D5 has been determined at 90.5 and 95°C respectively using the buffer as described above at a 1 mM Ca^{2+} concentration. It can be concluded that the thermal stability of both D7 and 2D5 is greatly increased (up to twofold for 2D5) over the entire pH range. (Figures 13 and 14).

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Example 9Production of mutant enzymes in Bacillus

5 Mutations in the B. licheniformis α -amylase, which
were identified by expression in E. coli WK6 were transferred
to a Bacillus expression vector in two different ways.

10 a) With the aid of the unique restriction sites within
the α -amylase gene (Figure 4), fragments carrying
mutations were isolated from pMatLia6 mutants and
subcloned into the homologous position of pBma6.Lia6.
The latter plasmid, which can be replicated either in
15 E. coli or in Bacillus, was subsequently digested with
SacI and recircularized with T4 DNA ligase. After
transformation into Bacillus subtilis 1A40 high level
 α -amylase production under control of the SPO₂ promoter
was obtained. Recircularized pBma6.Lia6 is named
20 pB6.Lia6 to indicate the removal of the E. coli
portion of the vector.

25 b) pBma6.Lia6 single stranded DNA was recollected from E.
coli and annealed with restriction enzyme digested
pBmc6.Lia6 double stranded DNA in order to obtain a
gapped duplex with the intended gap on the α -amylase
gene. This gap was then subjected to site-directed
mutagenesis with an oligonucleotide (as described in
the experimental section) which encodes the desired
mutation. pBmc6.Lia6 vector is then transformed into
30 pB6.Lia6 type vector as described above. Combination
of different single site mutation can be performed by
method a) if mutations are in different gaps,
preferably, however, method b) is used.

35 The mutations of mutants D7 and 2D5 were transferred
to pBma6.Lia6 by method a) by exchanging the SacII-SalI
fragments and α -amylase was recovered from the medium of

- 33 -

transf rmed Bacillus subtilis 1A40. Supernatants of both mutants were subjected to the screening procedures of Examples and it was confirmed that both mutants produce α -amylase which is more acid stable and more thermostable than
5 α -amylase produced by wild-type pB6.Lia6.

The phenotype of the α -amylase mutations in Bacillus is thus not different from the phenotype in E. coli.

Ultimately pB6.Lia6 mutants have been transformed into Bacillus licheniformis T9, which is a protease negative, α -
10 amylase negative derivative of Bacillus licheniformis T5, (EP-0253455, CBS 470.83). Host T9 has been used to produce high level amounts of α -amylase mutants in a homologous system. The removal of the chromosomal α -amylase gene renders this strain very suited for the production of mutant α -
15 amylase as no contaminating wild-type α -amylase is being produced anymore. Enzyme recovered from this strain has been used for industrial application testing. The industrial use of mutants pB6.Lia6.2D5 and pB6.Lia6.D7 was demonstrated.

20

Example 10

Application test of mutant α -amylase under conditions of starch liquefaction

25

To test mutant α -amylase 2D5 in more realistic circumstances, we have purified the fermentation broth (of Example 9) with ultrafiltration and formulated the enzyme with 50% propylene glycol.

30 Three samples have been tested:

893701 : WT	<u>B.licheniformis</u> T5 α -amylase	1530 TAU/g
893703 : 2D5	Mutant prepared as WT	2820 TAU/g
Maxamyl 0819	Commercial sample	7090 TAU/g

35 One TAU (thermostable α -amylase unit) is defined as the quantity of enzyme that will convert under standardized conditions 1 mg of starch per minute in a product having an

- 34 -

equal absorption to a reference colour at 620 nm after reaction with iodine. Standard conditions are pH 6.6; 30°C; reaction time : 20 min. Reference colour is 25g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 3.84 g $\text{K}_2\text{Cr}_2\text{O}_7$ and 1 ml HCl (1M) in 100 ml distilled H_2O .

5

1. Liquefaction test at low pH (5.5 and 5.25)

The temperature of starch slurry is increased to $110 \pm 0.5^\circ\text{C}$ as quick as possible and kept at this temperature for 6 minutes.

The liquefaction is realized in continuous flow (5.4 l/h). 3 Samples of 135 ml (1.5 minute of liquefaction) are taken after 45, 60 and 75 minutes of liquefaction and kept at 95°C for two hours. After this time, 50 ml of the sample are acidified with 0.4 ml H_2SO_4 N to obtain pH 3.5 and put in boiling bath for 10 minutes in order to stop enzymatic activity before D.E. determination.

The remaining part of the sample is cooled in order to determine residual enzymatic activity.

Slurry composition:

3.3 kg corn starch D.S. 88% (2.904 kg dry starch).

5.45 l well water (40 T.H.).

Dry substance of the slurry is 33%.

pH is corrected at 5.5 with 1N sulfuric acid or 1N NaOH.

Enzyme concentration: 4.4 TAU/gr dry starch.

The flow rate is verified two or three times during the trial.

30

2. Determination of D.E.

Dry substance of liquefied starch is verified with a refractometer (about 34%). D.E. is determined with the well-known Lane Eynon method. The results are shown in Figure 15.

- 35 -

3. Residual Enzymatic Activity

Residual amylase activity in liquefied starch is determined with a Brabender amylograph.

5

40 g potato starch
390 ml distilled water at 50°C
50 ml Tris buffer 0.05 M pH 6.50
5 ml $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at 30 g/l

10

The temperature is increased to 80°C (1.5°/min) when viscosity is stabilized (10 min) 5 ml of diluted liquefied starch (7 g up to 50 ml with distilled water) is added, the decrease of viscosity after 20 minutes is measured, this decrease is a function of the enzymatic activity. A standard curve with known enzymatic concentration allows to estimate residual activity in T.A.U.

Mutant 2D5 performs significantly better at pH < 5.5 and 110°C than WT enzyme. An improvement of 2-3 DE units at pH 5.25 is obtained with mutant 2D5.

Example 1125 Application test of mutant α -amylase under conditions of textile desizing

To test the industrial application of alkaline α -amylase mutants a test is performed on the stability at 20°C in the following solution:

	1.4%	H_2O_2 (35%)
	1.0-1.5%	Caustic Soda (100%)
	15-20 ml/l	Sodium Silicate (38 Bé)
35	0.3-0.5%	Alkylbenzene sulphonate (Lanaryl N.A.-ICI)
	0.5-1.0%	Organic stabilizer (Tinoclarite G)

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After incubation during 2.5 hours the α -amylase mutants selected for their desired properties should have any remaining enzyme activity.

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CLAIMS

1. A mutant α -amylase, that is the expression product of a mutated DNA sequence encoding an α -amylase,
5 characterized in that the mutant α -amylase has an amino acid sequence which differs at least in one amino acid from the wild-type enzyme and that said mutant α -amylase exhibits improved properties for application in the degradation of starch and/or textile desizing wherein the improved
10 properties are due to the amino acid replacements.
2. An α -amylase according to Claim 1, characterized in that it exhibits improved thermostability.
- 15 3. An α -amylase according to Claim 1, characterized in that it exhibits improved stability at a pH below 6.5 and/or above 7.5.
4. An α -amylase according to Claim 1, characterized in
20 that it exhibits improved thermostability and acid stability.
5. An α -amylase according to any one of the Claims 1-4, in which the original gene from which the mutant enzyme is derived is obtained from a microorganism, preferably a
25 Bacillus strain.
6. An α -amylase according to Claim 5, in which said gene is derived from a wild-type gene of a strain selected from the group consisting of B. stearothermophilus, B.
30 licheniformis and B. amyloliquefaciens.
7. An α -amylase according to Claim 6, characterized in that this enzyme differs from the wild-type α -amylase obtainable from Bacillus licheniformis by an amino acid
35 replacement at one or more of the positions 111, 133 and 149 or at corresponding positions in any homologous α -amylase.

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8. An α -amylase according to Claim 7, characterized in that it contains one or more of the following amino acid replacements: Ala-111-Thr, His-133-Tyr, Thr-149-Ile.

5 9. A mutant gene encoding an α -amylase as defined in any one of Claims 1-8.

10 10. An expression vector which comprises a mutant gene according to Claim 9.

11. A host cell harboring an expression vector according to Claim 10.

12. A host cell which is substantially incapable of
15 producing extracellular amylolytic enzymes prior to transformation, characterized in that it is transformed with an expression vector according to Claim 10.

13. A host cell according to Claim 12 being B. licheniformis T9.
20

14. A Bacillus/E. coli shuttle vector, wherein the expression of the cloned gene in E. coli is made impossible by physical separation of the regulatory sequences from the
25 structural gene and wherein the expression of the cloned gene in Bacillus can be restored by digestion with a single restriction enzyme and subsequent recircularization.

15. A method for preparing an amylolytic enzyme having
30 improved properties for application in starch degradation or in textile desizing which comprises the following steps:
mutagenizing a cloned gene encoding an amylolytic enzyme of interest or a fragment thereof;
isolating the obtained mutant amylase gene or genes;
35 introducing said mutant amylase gene or genes into a suitable host strain for expression and production;

recovering the produced mutant amylase and identifying those mutant amylases having improved properties for application in starch degradation or textile desizing.

- 5 16. A process for producing a mutant α -amylase comprising;
- cultivating a host cell according to any of Claims 11-13 in a suitable medium,
 - recovering the produced α -amylase.
- 10 17. Use of the α -amylase according to any one of the Claims 1-8 in starch degradation and in textile desizing.
18. Process for the degradation of starch which
- 15 comprises the use of a mutated α -amylase according to any one of the Claims 1-8.
19. Process for textile desizing which comprises the use of a mutated α -amylase according to any one of the Claims
- 20 1-8.
20. Starch degradation composition comprising a mutated α -amylase according to any one of the Claims 1-8.
- 25 21. Textile desizing composition comprising a mutated α -amylase according to any one of the Claims 1-8.

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10 20 30 40 50 60
AATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTT

70 80 90 100 110 120
TTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCAAGCTAATTCACCTC

130 140 150 160 170 180
GAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTTTTTTTGGAG

190 200 210 220 230 240
ATTTTCAACGTGAAAAAATTATTATTCGCAATTCCAAGCTCTGCCTCGCGCGTTTCGGTG

250 260 270 280 290 300
ATGACGGTGAAAACTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAG

310 320 330 340 350 360
CGGATGCAGATCACGCGCCCTGTAGCGCGCATTAAAGCGCGGGGGTGTGGTGGTTACGC

370 380 390 400 410 420
GCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCTT

430 440 450 460 470 480
CCTTTCTCGCCACGTTTCGCCGGCTTTCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAG

490 500 510 520 530 540
GGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTT

550 560 570 580 590 600
CACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTTCGCCCTTTGACGTTGGAGTCCACGT

610 620 630 640 650 660
TCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT

670 680 690 700 710 720
CTTTTGATTTATAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTT

730 740 750 760 770 780
AACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTGATCTGCGCTCG

790 800 810 820 830 840
GTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACA

850 860 870 880 890 900
GAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAAC

910 920 930 940 950 960
CGTAAAAAGGCCGCTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCAC

970 980 990 1000 1010 1020
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCG

1030 1040 1050 1060 1070 1080
TTTCCCCCTGGAAGCTCCCTCGTGGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATAC

1090 1100 1110 1120 1130 1140
CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTACGCTGTAGGTAT

Figure 1

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1150 1160 1170 1180 1190 1200
CTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAG

1210 1220 1230 1240 1250 1260
CCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGAC

1270 1280 1290 1300 1310 1320
TTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGT

1330 1340 1350 1360 1370 1380
GCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGT

1390 1400 1410 1420 1430 1440
ATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGC

1450 1460 1470 1480 1490 1500
AAACAAACCACCGCTGGTAGCGGTGTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGA

1510 1520 1530 1540 1550 1560
AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC

1570 1580 1590 1600 1610 1620
GAAAACTCACGTTAAGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC

1630 1640 1650 1660 1670 1680
CTTTTAAATTAATAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCT

1690 1700 1710 1720 1730 1740
GACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTCA

1750 1760 1770 1780 1790 1800
TCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCT

1810 1820 1830 1840 1850 1860
GGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACC GGCTCCAGATTTATCAGCA

1870 1880 1890 1900 1910 1920
ATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCC

1930 1940 1950 1960 1970 1980
ATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTG

1990 2000 2010 2020 2030 2040
CGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCT

2050 2060 2070 2080 2090 2100
TCATTTCAGCTCCGGTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAA

2110 2120 2130 2140 2150 2160
AAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTGAGAAGTAAGTTGGCCGAGTGTTA

2170 2180 2190 2200 2210 2220
TCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGC

2230 2240 2250 2260 2270 2280

Fig. 1 (continued)

TTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG
2290 2300 2310 2320 2330 2340
AGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAA
2350 2360 2370 2380 2390 2400
GTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAACTCTCAAGGATCTTACCGCTGTTG
2410 2420 2430 2440 2450 2460
AGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTC
2470 2480 2490 2500 2510 2520
ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGG
2530 2540 2550 2560 2570 2580
GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCAGACAG
2590 2600 2610 2620 2630 2640
TTTTATTGTTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGA
2650 2660 2670 2680 2690 2700
CACAACGTGGCTTTTGTTGAATAAATCGAACTTTTGCTGAGTTGACTCCCCGCGCGCATG
2710 2720 2730 2740 2750 2760
GGTCGAATTTGCTTTTCGAAAAAAGCCCGCTCATTAGGCGGGCTAAAAAAGCCCGCT
2770 2780 2790 2800 2810 2820
CATTAGGCGGGCTCGAATTTCTGCCATTTCATCCGTTATTATCACTTATTAGGCGTAGC
2830 2840 2850 2860 2870 2880
AACCAGGCGTTTAAGGGCACCAATAACTGCCTTAAAAAATTACGCCCGCCCTGCCACT
2890 2900 2910 2920 2930 2940
CATCGCACTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAGACGG
2950 2960 2970 2980 2990 3000
CATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCCTGCGTATAATATTGCG
3010 3020 3030 3040 3050 3060
CCATAGTGAAAACGGGGGCGAAGAAGTTGTCCATATTCGCCACGTTTAAATCAAACTGG
3070 3080 3090 3100 3110 3120
TGAAACTCACCCAGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCCTTAGGGA
3130 3140 3150 3160 3170 3180
AATAGGCCAGGTTTTACCGTAACACGCCACATCTTGCGAATATATGTGTAGAACTGCC
3190 3200 3210 3220 3230 3240
GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA
3250 3260 3270 3280 3290 3300
CGGTGTACAAGGGTGAACACTATCCCATATCACCAGCTCACCCTCTTTCATTGCCATAC
3310 3320 3330 3340 3350 3360
GAAATTCGGATGAGCATTTCATCAGGCGGGCAAGAATGTGAATAAAGCCGGATAAACT

Fig. 1 (continued)

3370 3380 3390 3400 3410 3420
TGTGCTTATTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTAAACGGTCTGGT

3430 3440 3450 3460 3470 3480
TATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGG

3490 3500 3510 3520 3530 3540
ATATATCAACGGTGGTATATCCAGTGATTTTTTCTCCATTTAGCTTCCTTAGCTCCTG

3550 3560 3570 3580 3590 3600
AAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTCATTATGGTGAAAGT

3610 3620 3630 3640 3650 3660
TGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGCCAAAAGTTGGCCCAGGGCTTCC

3670 3680 3690 3700 3710 3720
CGGTATCAACAGGGACACCAGGATTTATTTATTCTGCGAAGTGATCTTCCGTCACAGGTA

3730 3740 3750 3760 3770 3780
TTTATTGGAAGACGAAAGGGCATCGCGCGGGGAATTCGCGGGGATCCGTCGACCTGCA

3790 3800
GCCAAGCTTGGTCTAGAGGTCGA

Fig. 1 (continued)

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10 20 30 40 50 60
 GTCTACAAACCCCTTAAAAACGTTTTTAAAGGCTTTTAAGCCGCTGTACGTTCTTAAG
 70 80 90 100 110 120
 GAATTCACACTGGCCTTGGTTAAGGTTAAGATGTGGACGGAATGGGTAAAGTGTAGTAAA
 130 140 150 160 170 180
 GTACAATTAATCGGGAGCTTAGATGTCCCTTCAACATCTTATATAGAAGGGAAGGTTGGC
 190 200 210 220 230 240
 AAATGGAAATTGAAAGAATTAACGAGCATACAGTAAATTTTATATGTTTACGGAGATA
 250 260 270 280 290 300
 TTGAAGATCGCGGTTTTGACAGAGAAGAAATTTGGTATAACCGTGAGCGCAGTGAAGAAC
 310 320 330 340 350 360
 TTTTCTGGGAAGTCATGGATGAAGTTCATGAAGAAGAGGAATTCGAGCTCGCCCGGGGAT
 370 380 390 400 410 420
 CCAAGGAGGTGATCTAGAGTCATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACG
 M K Q Q K R L Y A R L L T
 430 440 450 460 470 480
 CTGTTATTTGGCGTCATCTTCTTGCTGCCTCATTCTGCAGCAGCGGCGGCAATCTTAAT
 L L F A L I F L L P H S A A A A A N L N
 490 500 510 520 530 540
 GGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGT
 G T L M Q Y F E W Y M P N D G Q H W K R
 5
 550 560 570 580 590 600
 TTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACTGCCGCTGGATTCCCCCG
 L Q N D S A Y L A E H G I T A V W I P P
 25
 610 620 630 640 650 660
 GCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGTACGACCTTTATGATTTA
 A Y K G T S Q A D V G Y G A Y D L Y D L
 45
 670 680 690 700 710 720
 GGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAAGTACGGGCACAAAAGGAGAGCTGCAA
 G E F H Q K G T V R T K Y G T K G E L Q
 65
 730 740 750 760 770 780
 TCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAAC
 S A I K S L H S R D I N V Y G D V V I N
 85
 790 800 810 820 830 840
 CACAAAGGCGCGCTGATCGCACCGAAGATGTAACCGCGGTGAAGTCGATCCCGCTGAC
 H K G G A D A T E D V T A V E V D P A D
 105
 850 860 870 880 890 900
 CGCAACCGCGTAATTTTCAAGGAGAACCTAATTAAAGCCTGGACACATTTTCATTTCCG
 R N R V I S G E H L I K A W T H F H F P
 125
 910 920 930 940 950 960
 GGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTACCATTTTGACGGAACCGAT
 G R G S T Y S D F K W H W Y H F D G T D
 145

Figure 2

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970 980 990 1000 1010 1020
TGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGG
W D E S R K L N R I Y K F Q G K A W D W
165

1030 1040 1050 1060 1070 1080
GAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTATGCCGACATCGATTATGAC
E V S N E N G N Y D Y L M Y A D I D Y D
185

1090 1100 1110 1120 1130 1140
CATCCTGATGTCGCGAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAA
H P D V A A E I K R W G T W Y A N E L Q
205

1150 1160 1170 1180 1190 1200
TTGGACGGTTTCCGCTCTTGATGCTGTCAAACACATTAAATTTTCTTTTTCGGGGATTGG
L D G F R L D A V K H I K F S F L R D W
225

1210 1220 1230 1240 1250 1260
GTTAATCATGTGAGGAAAAACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAG
V N H V R E K T G K E M F T V A E Y W Q
245

1270 1280 1290 1300 1310 1320
AATGACTTGGGCGCGCTGGAAAACATTTGAACAAAACAAATTTTAAATCATTGAGTTT
N D L G A L E N Y L N K T N F N H S V F
265

1330 1340 1350 1360 1370 1380
GACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATG
D V P L H Y Q F H A A S T Q G G G Y D M
285

1390 1400 1410 1420 1430 1440
AGGAAATTGCTGAACGGTACGGTTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTGTC
R K L L N G T V V S K H P L K S V T F V
305

1450 1460 1470 1480 1490 1500
GATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAG
D N H D T Q P G Q S L E S T V Q T W F K
325

1510 1520 1530 1540 1550 1560
CCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATACCCTCAGGTTTCTACGGG
P L A Y A F I L T R E S G Y P Q V F Y G
345

1570 1580 1590 1600 1610 1620
GATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATT
D M Y G T K G D S Q R E I P A L K H K I
365

1630 1640 1650 1660 1670 1680
GAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCACAGCATGATTATTTTCGAC
E P I L K A R K Q Y A Y G A Q H D Y F D
385

1690 1700 1710 1720 1730 1740
CACCATGACATTGTTCGCTGGACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTG
H H D I V G W T R E G D S S V A N S G L
405

1750 1760 1770 1780 1790 1800
GCGGCATTAAATAACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAAC
A A L I T D G P G G A K R M Y V G R Q N
425

Fig. 2 (continued)

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1810 1820 1830 1840 1850 1860
GCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTTCG
A G E T W H D I T G N R S E P V V I N S
445
1870 1880 1890 1900 1910 1920
GAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGATAG
E G W G E F H V N G G S V S I Y V Q R
465 483
1930 1940 1950 1960 1970 1980
AAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTTTTTTTATTTTGCCCGTCTTATA
1990 2000 2010 2020 2030 2040
AATTTCTTTGATTACATTTTATAATTAATTTTAACAAAGTGTCATCAGCCCTCAGGAAGG
2050 2060 2070 2080 2090 2100
ACTTGCTGACAGTTTGAATCGCATAGGTAAGGCGGGGATGAAATGGCAACGTTATCTGAT
2110 2120 2130 2140
GTAGCAAAGAAAGCAAATGTGTGCGAAAATGACGGTATCGCGGGTGATCA

Fig. 2 (continued)

10 20 30 40 50 60
AATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTT

70 80 90 100 110 120
TTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCAAGCTAATTCACCTC

130 140 150 160 170 180
GAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTTTTTTTGGAG

190 200 210 220 230 240
ATTTTCAACGTGAAAAAATTATTATTCGCAATTCCAAGCTCTGCCTCGCGCGTTTCGGTG

250 260 270 280 290 300
ATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAG

310 320 330 340 350 360
CGGATGCAGATCACGCGCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGTTACGC

370 380 390 400 410 420
GCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTT

430 440 450 460 470 480
CCTTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAG

490 500 510 520 530 540
GGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTT

550 560 570 580 590 600
CACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTTCGCCCTTTGACGTTGGAGTCCACGT

610 620 630 640 650 660
TCTTTAATAGTGGACTCTTGTTCCAACTGGAACAACACTCAACCCTATCTCGGTCTATT

670 680 690 700 710 720
CTTTTGATTTATAAGGGATTTTGGCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTT

730 740 750 760 770 780
AACAAAAATTTAACGCGAATTTTAACAAAATATTAAACGTTTACAATTTGATCTGCGCTCG

790 800 810 820 830 840
GTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTATCCACA

850 860 870 880 890 900
GAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAAC

910 920 930 940 950 960
CGTAAAAAGGCCGCGTTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCAC

970 980 990 1000 1010 1020
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCC

1030 1040 1050 1060 1070 1080
TTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATAC

1090 1100 1110 1120 1130 1140
CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTAT

Figure 3

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1150 1160 1170 1180 1190 1200
CTCAGTTCGGTGTAGGTCGTTCCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAG

1210 1220 1230 1240 1250 1260
CCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGAC

1270 1280 1290 1300 1310 1320
TTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGT

1330 1340 1350 1360 1370 1380
GCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGT

1390 1400 1410 1420 1430 1440
ATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA AAAAGAGTTGGTAGCTCTTGATCCGGC

1450 1460 1470 1480 1490 1500
AAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTGCAAGCAGCAGATTACGCGCAGA

1510 1520 1530 1540 1550 1560
AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC

1570 1580 1590 1600 1610 1620
GAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC

1630 1640 1650 1660 1670 1680
CTTTTAAATTAAAAATGAAGTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCT

1690 1700 1710 1720 1730 1740
GACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTCA

1750 1760 1770 1780 1790 1800
TCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCT

1810 1820 1830 1840 1850 1860
GGCCCCAGTGCTGCAATGATACCGCGAGACCCAGGCTCACCGGCTCCAGATTTATCAGCA

1870 1880 1890 1900 1910 1920
ATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAAGTGGTCCTGCAACTTTATCCGCCTCC

1930 1940 1950 1960 1970 1980
ATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTG

1990 2000 2010 2020 2030 2040
CGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCT

2050 2060 2070 2080 2090 2100
TCATTACGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAA

2110 2120 2130 2140 2150 2160
AAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCGAGTGTTA

2170 2180 2190 2200 2210 2220
TCACTCATGGTTATGGCAGCACTGCATAATTCTTACTGTATGCCATCCGTAAGATGC

2230 2240 2250 2260 2270 2280

Fig. 3 (continued)

TTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG
2290 2300 2310 2320 2330 2340
AGTTGCTCTTGCCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAA
2350 2360 2370 2380 2390 2400
GTGCTCATCATTTGAAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTG
2410 2420 2430 2440 2450 2460
AGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTC
2470 2480 2490 2500 2510 2520
ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG
2530 2540 2550 2560 2570 2580
CGGACACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCAGACAG
2590 2600 2610 2620 2630 2640
TTTTATTGTTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGA
2650 2660 2670 2680 2690 2700
CACAACGTGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGACTCCCCGCGCGCATG
2710 2720 2730 2740 2750 2760
GGTCGAATTTGCTTTCGAAAAAAAGCCCGCTCATTAGGCGGGCTAAAAAAAGCCCGCT
2770 2780 2790 2800 2810 2820
CATTAGGCGGGCTCGAATTTCTGCCATTTCATCCGCTTATTATCACTTATTCAGGCGTAGC
2830 2840 2850 2860 2870 2880
AACCAGGCGTTTAAGGGCACCAATAACTGCCTTAAAAAATTACGCCCCGCCCTGCCACT
2890 2900 2910 2920 2930 2940
CATCGCAGTACTGTTGTAATTCATTAAAGCATTCTGCCGACATGGAAGCCATCACAGACGG
2950 2960 2970 2980 2990 3000
CATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTC
3010 3020 3030 3040 3050 3060
CCATAGTGAAAACGGGGGCGAAGAAGTTGTCCATATTCGCCACGTTTAAATCAAACTGG
3070 3080 3090 3100 3110 3120
TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCCTTTAGGGA
3130 3140 3150 3160 3170 3180
AATAGGCCAGGTTTTACCGTAACACGCCACATCTTGCGAATATATGTGTAGAACTGCG
3190 3200 3210 3220 3230 3240
GGAAATCGTTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA
3250 3260 3270 3280 3290 3300
CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCCTCTTTCATTGCCATAC
3310 3320 3330 3340 3350 3360
GAAATTCGGATGAGCATTTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAACT

Fig. 3 (continued)

3370 3380 3390 3400 3410 3420
TGTCCTTATTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTAAACGGTCTGGT

3430 3440 3450 3460 3470 3480
TATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGG

3490 3500 3510 3520 3530 3540
ATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTG

3550 3560 3570 3580 3590 3600
AAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTTTATTATGGTGAAAGT

3610 3620 3630 3640 3650 3660
TGGAACCTCTTACGTGCCGATCAACGTCTCATTTTTCGCCAAAAGTTGGCCAGGGCTTCC

3670 3680 3690 3700 3710 3720
CGGTATCAACAGGGACACCAGGATTTATTTATTCTGCGAAGTGATCTTCCGTCACAGGTA

3730 3740 3750 3760 3770 3780
TTTATTGGAAGACGAAAGGGCATCGCGCGGGGAATTCGAGCTCGAGCTTACTCCCCAT

3790 3800 3810 3820 3830 3840
CCCCCTGTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAA

3850 3860 3870 3880 3890 3900
TTTCACACAGGAAACAGGATCCAAGGAGGTGATCTAGAGTCATGAAACAACAAAAACGGC
M K Q Q K R

3910 3920 3930 3940 3950 3960
TTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCTTGCTGCCTCATTTCTGCAG
L Y A R L L T L L F A L I F L L P H S A

3970 3980 3990 4000 4010 4020
CAGCGGCGGCAAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATG
A A A A N L N G T L M Q Y F E W Y M P N

+1
4030 4040 4050 4060 4070 4080
ACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTA
D G Q H W K R L Q N D S A Y L A E H G I

18
4090 4100 4110 4120 4130 4140
CTGCCGTCTGGATTCCCCCGCATATAAGGGAAGTCAAGCGGATGTGGGCTACGGTG
T A V W I P P A Y K G T S Q A D V G Y G

38
4150 4160 4170 4180 4190 4200
CTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACG
A Y D L Y D L G E F H Q K G T V R T K Y

58
4210 4220 4230 4240 4250 4260
GCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGGACATTACGTTT
G T K G E L Q S A I K S L H S R D I N V

78
4270 4280 4290 4300 4310 4320
ACGGGGATGTGGTCATCAACCACAAAGCGCGCTGATGCGACCGAAGATGTAACCGCGG
Y G D V V I N H K G G A D A T E D V T A

98

Fig. 3 (continued)

4330 4340 4350 4360 4370 4380
TTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTACAGGAGAACACCTAATTAAAGCCT
V E V D P A D R N R V I S G E H L I K A
118

4390 4400 4410 4420 4430 4440
GGACACATTTTCATTTTCCGGGGCGCGCAGCACATACAGCGATTTTAAATGGCATTTGGT
W T H F H F P G R G S T Y S D F K W H W
138

4450 4460 4470 4480 4490 4500
ACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTC
Y H F D G T D W D E S R K L N R I Y K F
158

4510 4520 4530 4540 4550 4560
AAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGT
Q G K A W D W E V S N E N G N Y D Y L M
178

4570 4580 4590 4600 4610 4620
ATGCCGACATCGATTATGACCATCCTGATGTGCGCAGCAGAAATTAAGAGATGGGGCACTT
Y A D I D Y D H P D V A A E I K R W G T
198

4630 4640 4650 4660 4670 4680
GGTATGCCAATGAACTGCAATTGGACGGTTTCCGCTCTTGATGCTGTCAAACACATTAAAT
W Y A N E L Q L D G F R L D A V K H I K
218

4690 4700 4710 4720 4730 4740
TTTCTTTTTTGGCGGATTGGGTTAATCATGTGAGGAAAAACGGGGAAGGAAATGTTTA
F S F L R D W V N H V R E K T G K E M F
238

4750 4760 4770 4780 4790 4800
CGGTAGCTGAATATTGGCAGAATGACTTGGGCGCCCTGGAAAACTATTTGAACAAACAA
T V A E Y W Q N D L G A L E N Y L N K T
258

4810 4820 4830 4840 4850 4860
ATTTTAATCATTGAGTGTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACAC
N F N H S V F D V P L H Y Q F H A A S T
278

4870 4880 4890 4900 4910 4920
AGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTTCGTTTCCAAGCATCCGT
Q G G G Y D M R K L L N G T V V S K H P
298

4930 4940 4950 4960 4970 4980
TGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGA
L K S V T F V D N H D T Q P G Q S L E S
318

4990 5000 5010 5020 5030 5040
CTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGAT
T V Q T W F K P L A Y A F I L T R E S G
338

5050 5060 5070 5080 5090 5100
ACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTC
Y P Q V F Y G D M Y G T K G D S Q R E I
358

5110 5120 5130 5140 5150 5160
CTGCCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATCGGTACGGAG
P A L K H K I E P I L K A R K Q Y A Y G
378

Fig. 2 (continued)

5170 5180 5190 5200 5210 5220
CACAGCATGATTATTTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCT
A Q H D Y F D H H D I V G W T R E G D S
398
5230 5240 5250 5260 5270 5280
CGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAA
S V A N S G L A A L I T D G P G G A K R
418
5290 5300 5310 5320 5330 5340
TGTATGTCGGCCGGCAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGG
M Y V G R Q N A G E T W H D I T G N R S
438
5350 5360 5370 5380 5390 5400
AGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTTCGTTT
E P V V I N S E G W G E F H V N G G S V
458
5410 5420 5430 5440 5450 5460
CAATTTATGTTCAAAGATAGGTGACCAGAGAGGACGGATTTCTGAAGGAAATCCGTTT
S I Y V Q R
478
5470 5480 5490 5500 5510 5520
TTTATTTTGCCCGTCTTATAAATTTCTTTGATTACATTTTATAATTAATTTTAACAAAGT
5530 5540 5550 5560 5570 5580
GTCATCAGCCCTCAGGAAGGACTTGCTGACAGTTTGAATCGCATAGGTAAGGCGGGGATG
5590 5600 5610 5620 5630 5640
AAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAATGTGTGCGAAAATGACGGTATCGC
5650 5660 5670
GGGTGATCCTCTAGAAGAAGCTTGGTCTAGAGGTCGA

Fig. 3 (continued)

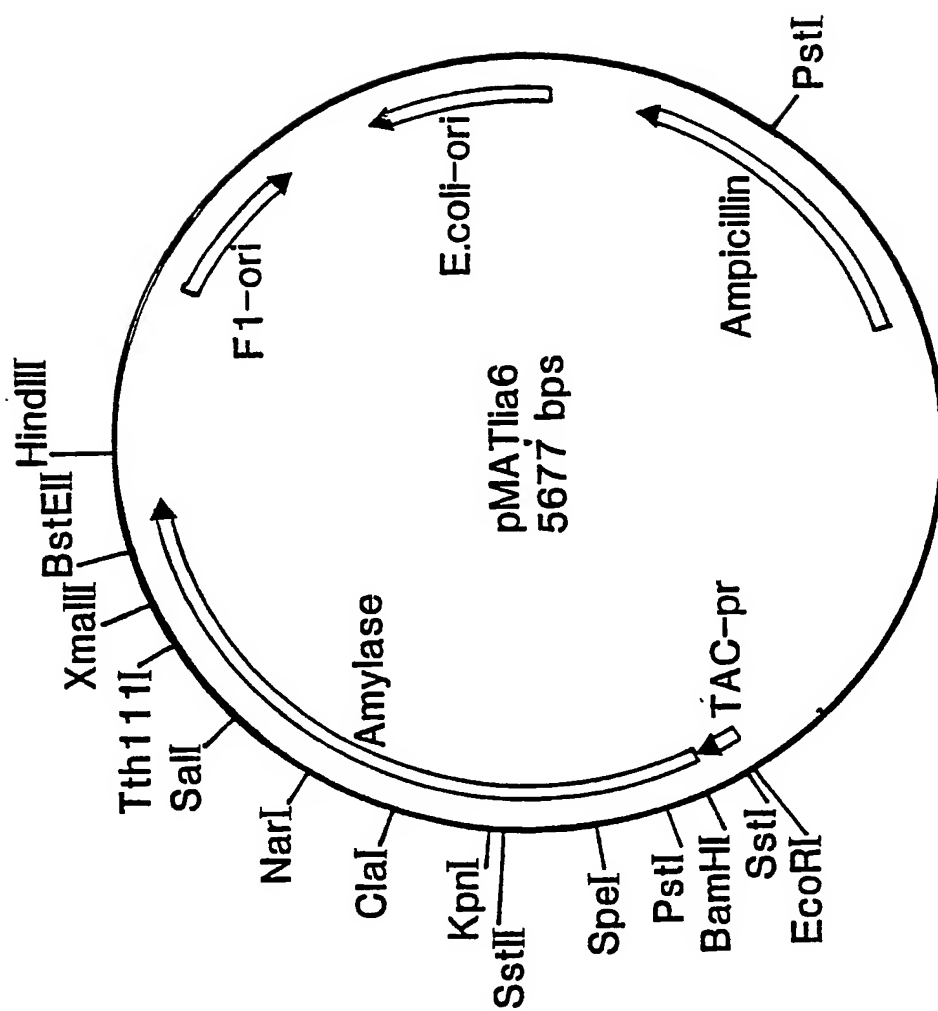


Figure 4

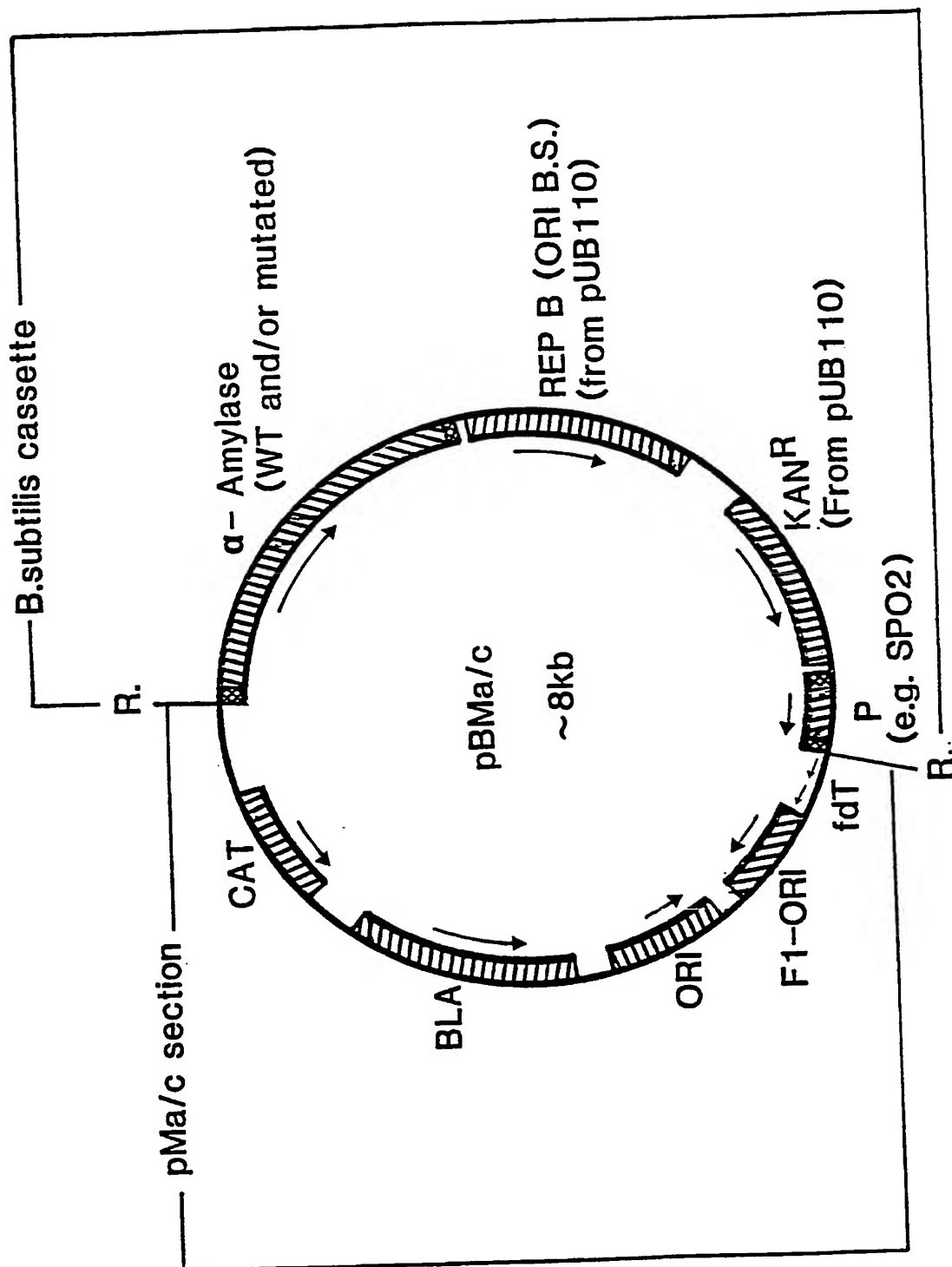


Figure 5

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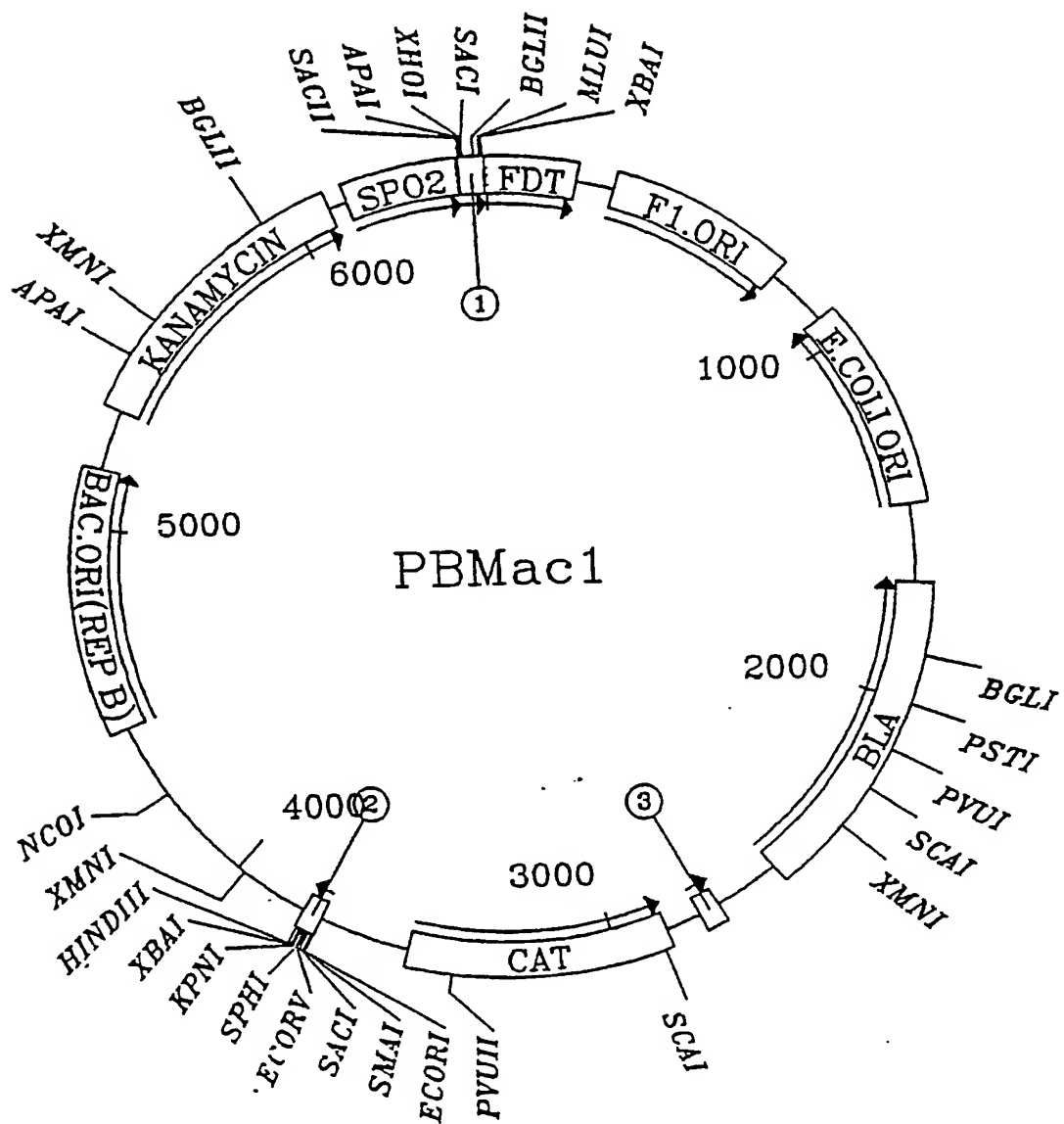


Figure 6

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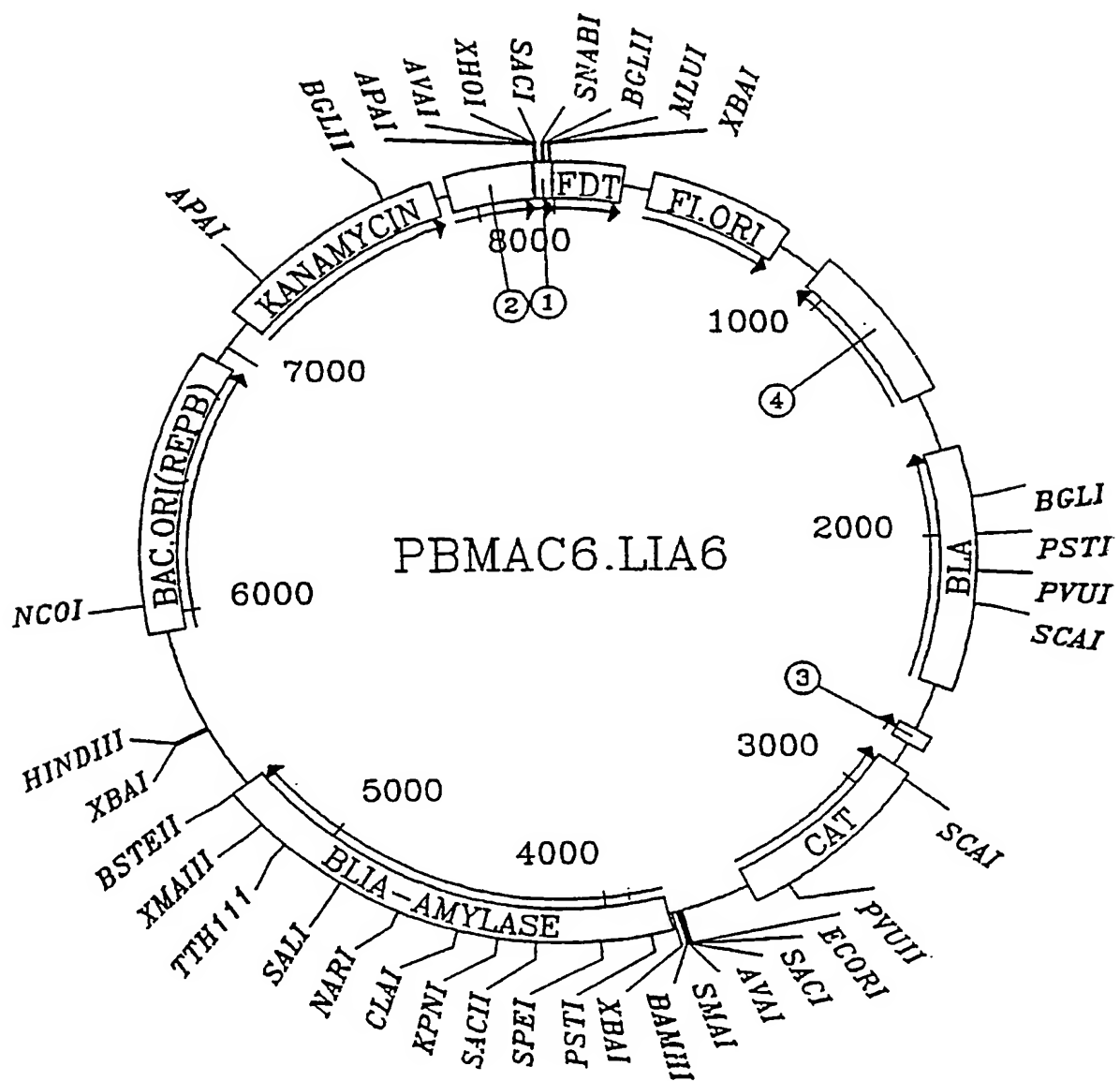


Figure 7

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EcoRI

GAATTCGAGCTCGAGCTTACTCCCCATCCCCCTGTTGACAATTAATCATCGGCTCGTATA
BamHI

ATGTGTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAGGATCCCGGGATCCGTG

----->phoA
GAGAAAATAAA GTGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACC
M K Q S T I A L A L L P L L F T

CCTGTGACAAAAGCG GCAAAT
P V T K A A N
----->amylase

Figure 8

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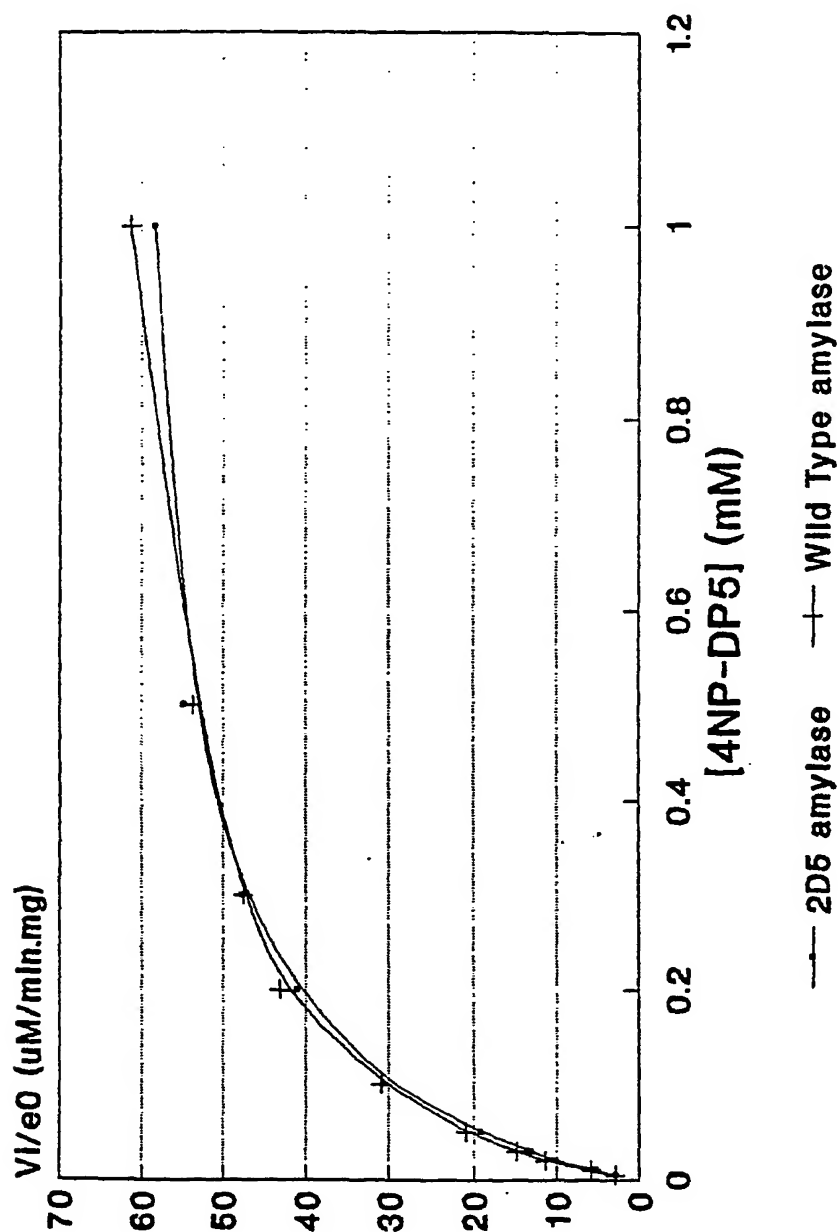


Figure 9

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Thermostoinactivation at pH 5.5

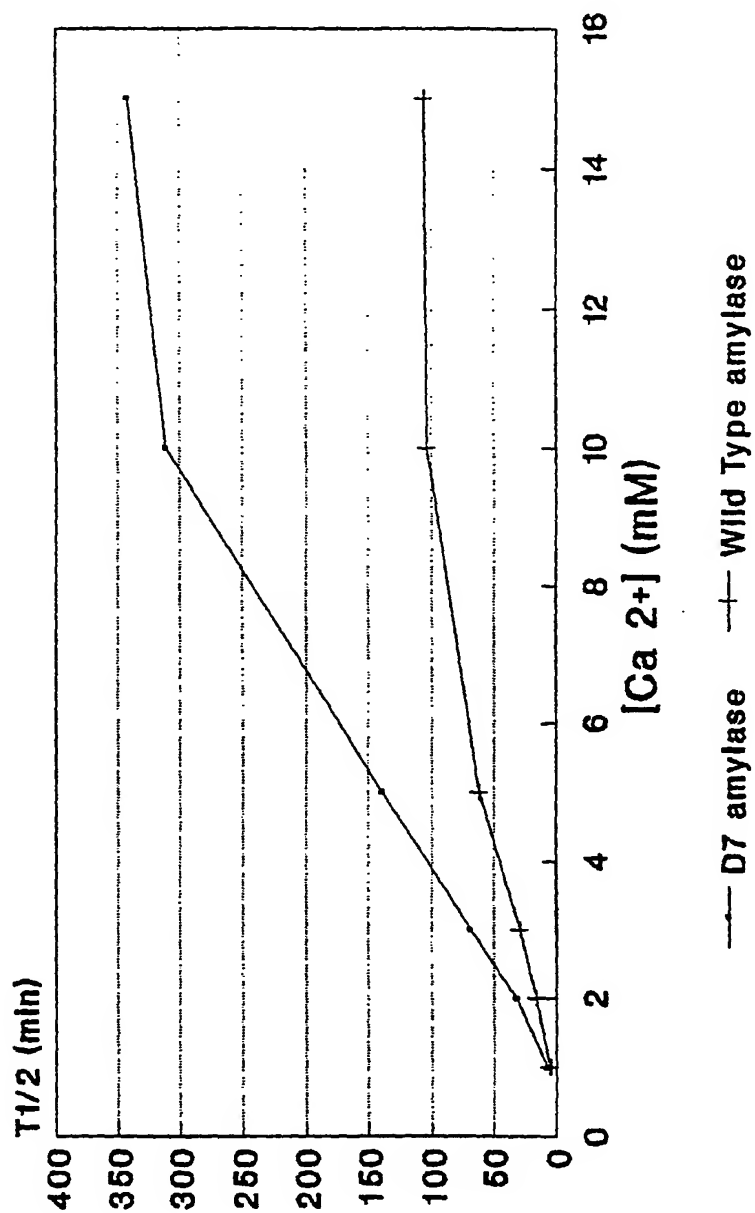


Figure 10

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Thermostoinactivation at pH 7.0

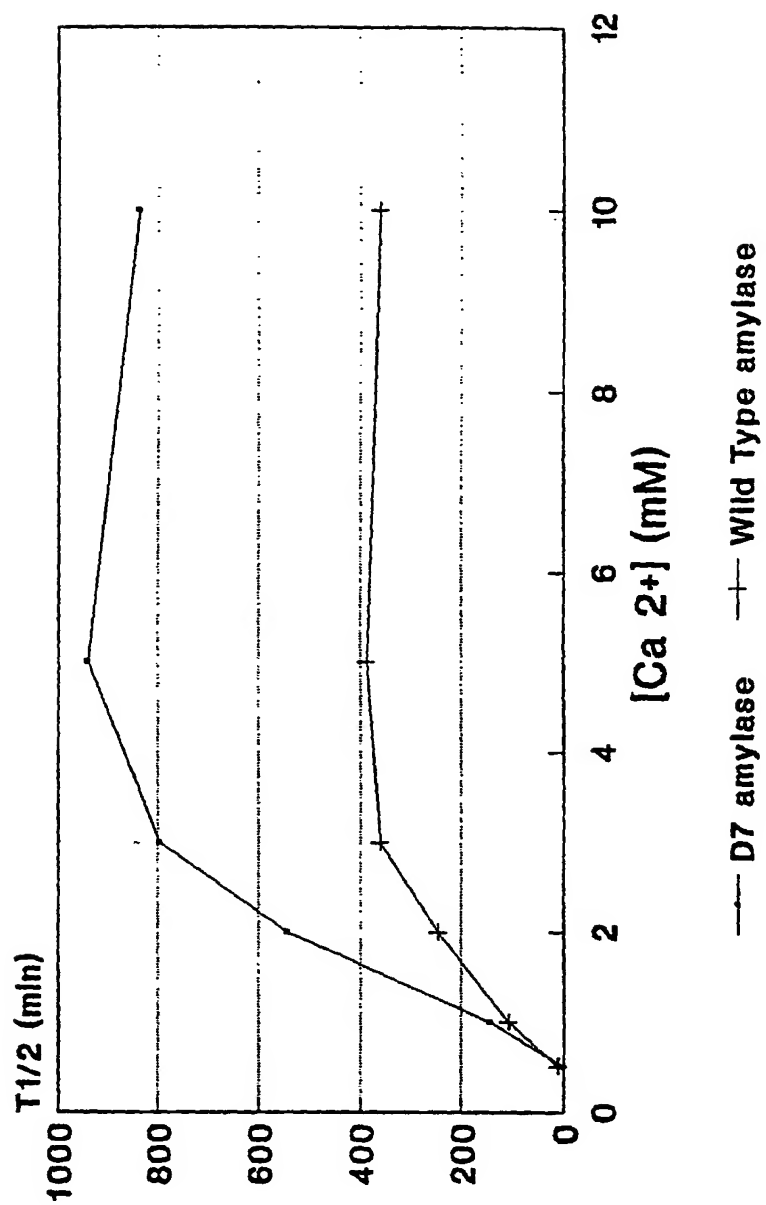


Figure 11

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Thermoinactivation of 2D5 amylase

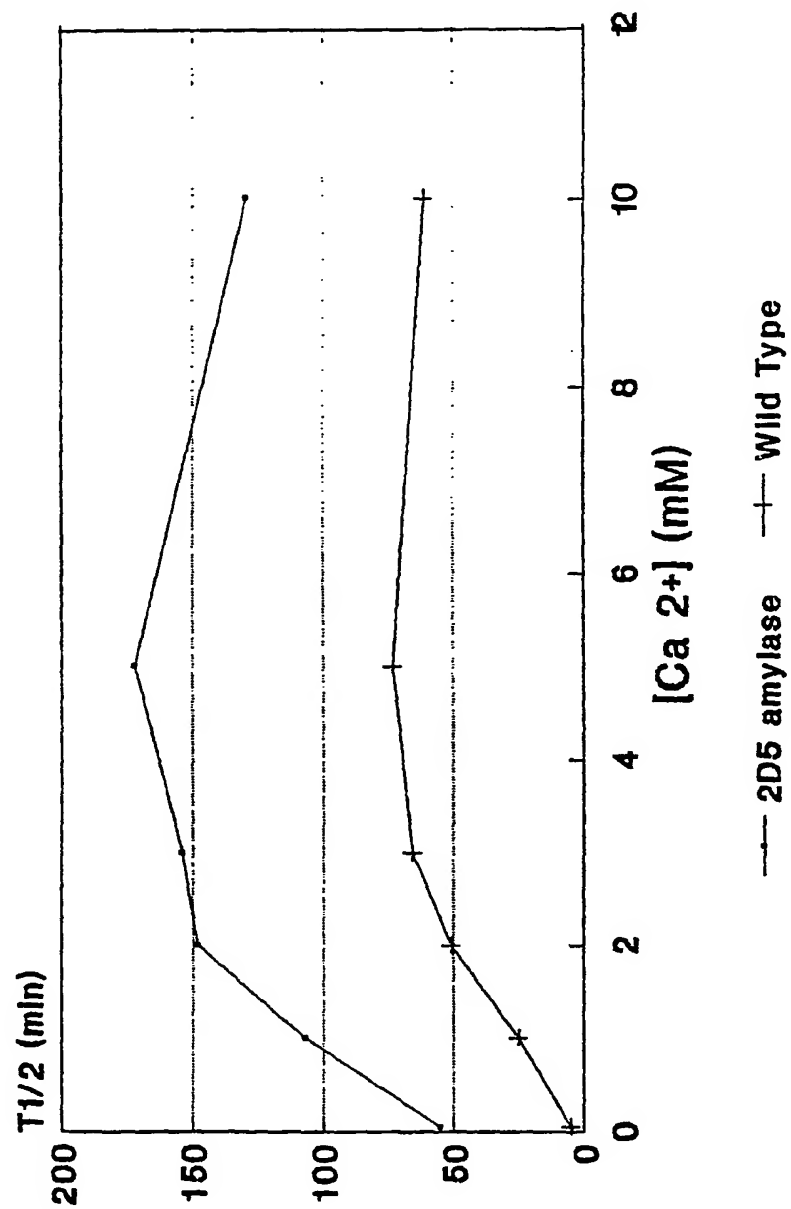


Figure 12

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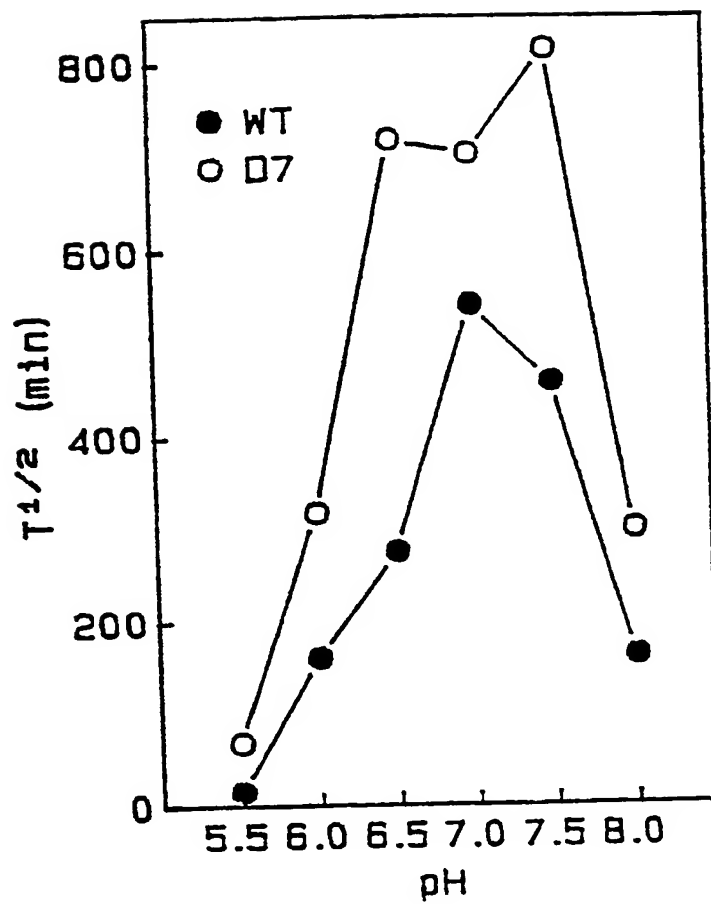


Figure 13

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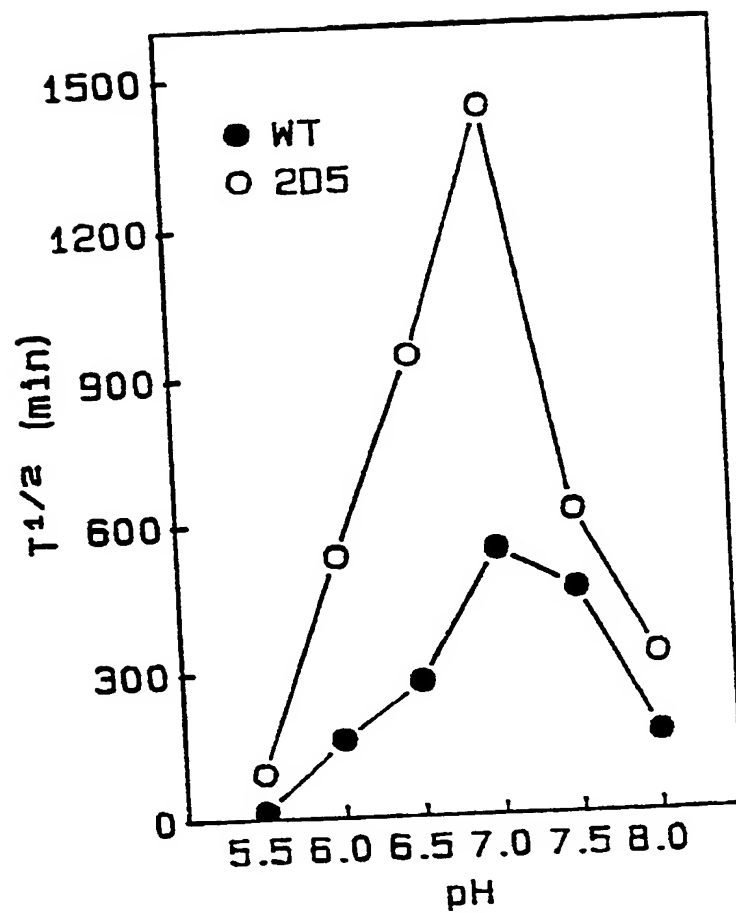


Figure 14

Liquefaction at 110 C

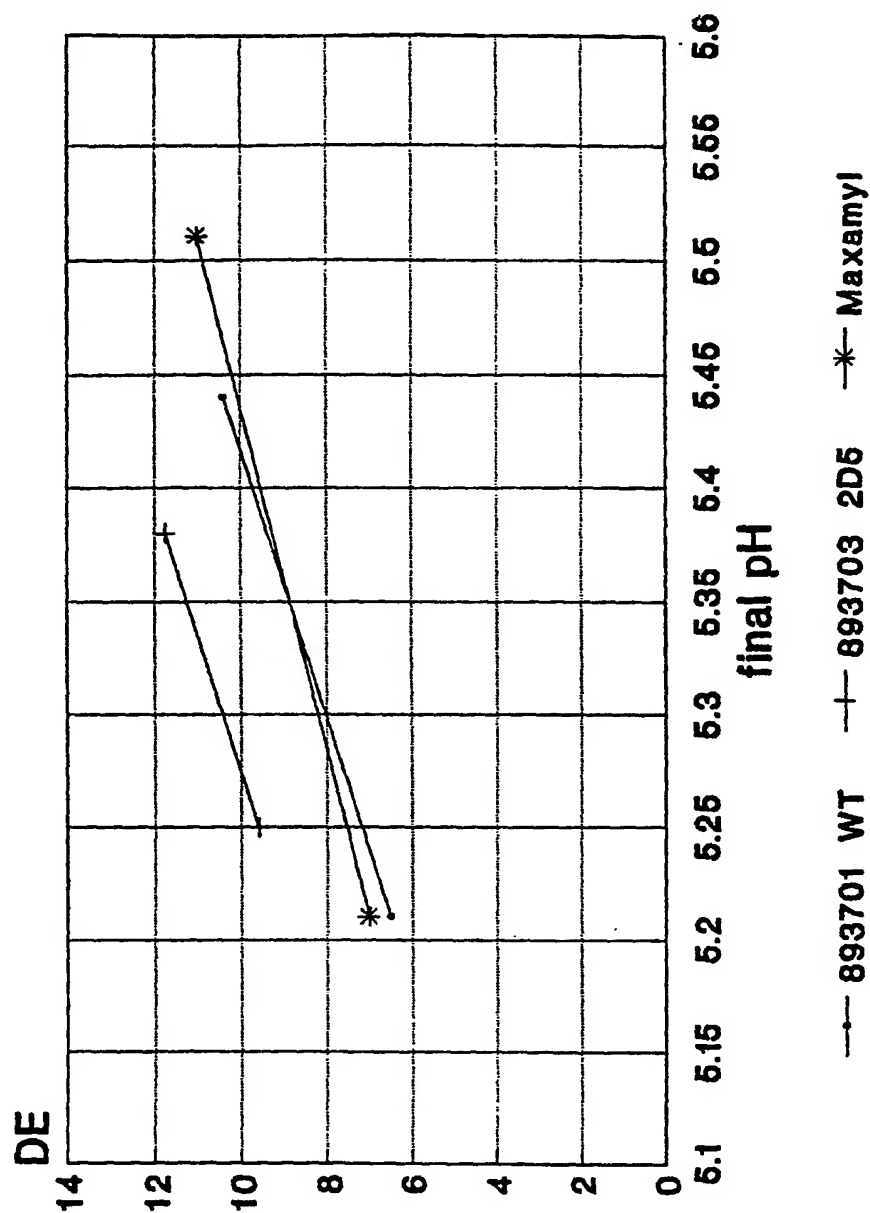


Figure 15

L1 ANSWER 1 OF 1 WPIDS COPYRIGHT 2001 DERWENT INFORMATION LTD
ACCESSION NUMBER: 2001-300800 [32] WPIDS
DOC. NO. CPI: C2001-092471
TITLE: Novel low temperature alkaline protease and its production
method, use and microbe for producing the protease.
DERWENT CLASS: D13 D16 D21 D25
INVENTOR(S): SUN, M; WANG, Y; ZHANG, Y
PATENT ASSIGNEE(S): (HUAN-N) HUANGHAI AQUATIC PROD INST CHINA AQUATIC
COUNTRY COUNT: 1
PATENT INFORMATION:

PATENT NO	KIND	DATE	WEEK	LA	PG
CN 1280186	A	20010117	(200132)*		<--

APPLICATION DETAILS:

PATENT NO	KIND	APPLICATION	DATE
CN 1280186	A	CN 2000-123404	20000815

PRIORITY APPLN. INFO: CN 2000-123404 20000815